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*THE ENZYMATIC SYNTHESIS IN VITRO OF HYALURONIC ACID CHAINS**

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INTRODUCTION

The structure of hyaluronic acid has been elucidated largely by the work of Meyer and collaborators,[1, 2, 3] who have shown it to be a linear polymer of alternating units of N-acetyl-D-glucosamine and D-glucuronic acid. The monosaccharide components appear to be combined in alternating β-1,3- and β-1,4-linkages, the former being those involving C_1 of GA[4] and the latter those involving C_1 of AG. Hyaluronic acid isolated from different tissues appears to have constant chemical composition[3, 5, 6] but varies in average molecular weight from 300,000 to 1,500,000.[7] No enzymatic synthesis of hyaluronic acid by cell-free preparations has been reported.

We have now been able to demonstrate the synthesis of oligosaccharide chains having the structure of hyaluronic acid by using cell-free homogenates of the Rous chicken sarcoma,[8] a tissue which has been known for a long time to produce large quantities of the mucopolysaccharide[6, 9, 10] and to have very little if any active hyaluronidase.[10] Synthesis of the oligosaccharides has been shown by incubating sarcoma homogenates with uridine diphospho-N-acetyl-glucosamine labeled with C^{14} in the N-acetyl group, together with uridine diphosphoglucuronic acid or with uridine diphosphoglucose and diphosphopyridine nucleotide. In some experiments the homogenates were incubated with uridine triphosphate and N-acetyl-glucosamine-6-phosphate labeled with C^{14} in the N-acetyl group. The preparation of the latter material with a specific yeast N-acetylase has been described.[11] UDPAg was discovered by Leloir and co-workers,[12, 13] but no coenzymatic function for it was demonstrated. UDPGA has been isolated and found to be the substrate for glucuronide synthesis by liver extracts.[14, 15] The formation of UDPGA by enzymatic oxidation of UDPG in the presence of DPN has been described by Kalckar and coworkers.[16] Evidence discussed below indicates that UDPAg and UDPGA are the substrates from which hyaluronic acid chains are synthesized.

MATERIALS AND METHODS

The C^{14}-UDPAg used was made enzymatically by incubating C^{14}-AG-6-P with UTP and a small amount of α-glucose-1,6-diphosphate[17] at pH 7.0 and 30° in the presence of Mg^{++} and an enzyme prepared by sonic disintegration[18] of baker's yeast (Fleishchmann), followed by protamine precipitation of nucleic acid and dialysis for 24 hours at 0° against 0.002 *M* EDTA, pH 6.7. The enzyme preparation contains the mutase, first studied by Leloir and Cardini,[19] which equilibrates AG-6-P and AG-1-P. We have confirmed their finding that its activity is stimulated by α-glucose-1,6-diphosphate. In addition to this mutase, our yeast extract contains the enzyme which synthesizes UDPAg by the following reaction, proceeding from left to right:

$$\text{AG-1-P} + \text{UTP} = \text{UDPAg} + \text{Inorg. pyrophosphate.} \quad (1)$$

The reversal of this reaction was first described by Smith and Munch-Petersen,[20] who found that UDPAg is pyrophosphorylated by an enzyme from rat liver nuclei to yield UTP and an unidentified second product. In the yeast extract which we have used, a stoichiometric yield of UDPAg from AG-6-P is obtained because of the presence of an inorganic pyrophosphatase which causes reaction (1) above to proceed to completion toward the right. Synthesis of C^{14}-UDPAg from C^{14}-AG-6-P has also been observed in the Rous sarcoma homogenates, where accumulation of the nucleotide has been found to occur under a variety of incubation conditions. UDPAg was identified following chromatography on Dowex-1 (formate) as a uridine derivative (ultraviolet spectrum) having one mole of acid-labile (10 minutes, pH 2, 100°) AG (modified Morgan and Elson test[21]). It migrated on a paper chromatogram (Whatman No. 1) in the neutral ammonium acetate–ethanol solvent of Paladini and Leloir[12] as a single spot, with $R_{\text{UMP-5}'} = 1.53$. Nonisotopic UDPAg was obtained from Sigma Chemical Company.

The UDPGA used was prepared enzymatically from UDPG (isolated from yeast or supplied by Sigma Chemical Company) by incubating it with DPN, according to Strominger *et al.*,[16] using an enzyme obtained by ammonium sulfate fractionation of an extract of pigeon liver acetone powder. It has been found that the sarcoma homogenates prepared as described below also contain the enzyme which oxidizes UDPG to UDPGA. The latter material has been isolated from incubation mixtures of the sarcoma homogenates by chromatography on Dowex-1 (formate) and identified subsequently by paper chromatography (as above) as a single spot having $R_{\text{UMP-5}'} = 0.76$. The material gave a glucuronic acid test according to Dische[22] and was active in the enzymatic glucuronide synthesis system of Dutton and Storey.[14]

Hyaluronic acid was isolated following addition of carrier (bovine vitreous humor HA, Worthington Biochemical Corporation) by a slight modification of the method of Harris, Malmgren, and Sylvén.[6] The resulting HA sedimented slowly in 0.04 *M* maleate buffer, pH 6.7, in the Spinco ultracentrifuge, showing a single peak with $S_{20} = 0.78$. The HA isolated from the Rous sarcoma has been reported to have an S of 1.0.[6] Hyaluronic acid was determined quantitatively as GA, using the method of Dische.[22]

Sarcomas were developed in the wing muscle of 6–8-week-old New Hampshire Red or White chickens. After 3 weeks of growth of the tumor, the chickens were

anesthetized with nembutal and bled from the neck, and the sarcomas were excised. They weighed 8–10 gm. each. Homogenates were prepared by cutting the sarcoma into small pieces with a scalpel and grinding the pieces in the Waring Blendor with twice their weight of pH 8.0 buffer (0.02 *M* THM–0.001 *M* EDTA–0.01 *M* $MgCl_2$) at 0° for four $^3/_4$-minute periods, with cooling in ice between. The mixture was centrifuged for 5 minutes at 10,000 × g, and the supernatant fluid was used.

EXPERIMENTAL RESULTS

Table 1 shows the details of experiments in which incorporation of C^{14} into material isolated with carrier HA is shown. Incorporation was obtained either from UDPAg or AG-6-P (experiments 6A and 6B). In experiment 6C the only source of uridine nucleotides was the tissue homogenate itself. Here incorporation of the isotope was not less than that observed in the experiments with added nucleotides. This finding raises the question of whether the pathway of incorporation of AG-6-P is via UDPAg. As is shown below, synthesis of C^{14}-HA is strictly dependent upon the presence of uridine nucleotides. Experiments 2A and 7 show that no incorporation of C^{14} is observed when enzymatic action is stopped at zero time by addition of ethanol. The HA from experiments 2A and 2B was dialyzed before assay. That from the other experiments was not. It has been shown that dialysis for 2 days at 5° against a large volume of water of any of the C^{14}-HA samples of Table 1 leads to a loss of from 50 to 70 per cent of the C^{14}, while only 10 to 20 per cent of the sample is lost. Electrodialysis for 48 hours at 800 volts in Visking casing removed almost all the isotope from any sample. The dialyzability of the label originally suggested that the C^{14} observed in the HA was due solely to

TABLE 1

INCORPORATION OF C^{14}-URIDINE DIPHOSPHO-N-ACETYL-GLUCOSAMINE OR C^{14}-N-ACETYL-GLUCOSAMINE-6-PHOSPHATE INTO HYALURONIC ACID BY HOMOGENATES OF ROUS CHICKEN SARCOMA

EXP. NO.	URIDINE NUCLEOTIDE ADDED (μMOLES)				C^{14}-AG-6-P** (μMOLES)	INCUBATION TIME (MIN)	HA ADDED BEFORE INCUBATION (MG)	CARRIER HA ADDED AFTER INCUBATION (MG)	HA ISOLATED (MG)	SP. ACT. OF HA (COUNTS/MIN/MG GA)	TOTAL C^{14} IN HA (COUNTS/MIN)
	UDP	UTP	UDPG	UDPGA	C^{14}-UDPAg*						
6A†	12	Trace	3.27	1.0	2.44	...	90	7.5	38.5	32.2	58.7	969
6B†	2.0	20.0	3.27	...	...	4.67	90	7.5	38.5	31.6	44.4	719
6C†	...	...	...	...	...	2.33	90	3.8	19.2	21.3	47.5	517
2A‡	...	...	...	...	...	2.33	0	28.5	0	15.1	0.0	0
2B‡	0.6	50.0	0.6	...	...	3.11	120	3.4	20.0	17.3	44.5	394
7	...	...	...	...	...	2.33	0	30.0	0	26.2	1.5	20

* Compound had 67,500 counts/min/μmole. C^{14} present in the carboxyl C of acetyl group.

† Reaction mixture (8 ml. in experiments 6A and 6B) contained DPN, 1.8×10^{-3} *M*; $MgCl_2$, 5×10^{-3} *M*; THM, 1×10^{-2} *M*; EDTA, 6×10^{-4} *M*; glutathione, 12 mg. In addition, experiments 6B and 6C contained 1.2×10^{-3} *M* α-glucose-1,6-diphosphate. In experiments 6A and 6B, enzyme: 6 ml. homogenate from 2 gm. tissue. In experiment 6C (4.5 ml.), enzyme: 3 ml. homogenate from 1 gm. tissue. pH maintained at 6.8–7.5, 37°.

‡ Reaction mixture (7.1 ml.) contained glutathione, $MgCl_2$, THM, and EDTA, as in experiment 6A. In addition, experiment 2B contained DPN, 1.1×10^{-3} *M*. Enzyme: 3.5 ml. homogenate from 0.9 gm. tissue. 37°.

contamination by one or more of the labeled compounds introduced or to labeled degradation products derived from them. This possibility was examined by reprecipitating a number of samples of C^{14}-HA from large molar excesses of all substances whose presence seemed likely. Table 2 shows the results of these experiments. The recovery of HA varied from 75 to 95 per cent, and the samples retained approximately 40 per cent of their C^{14} content. Although the loss of C^{14} was about twice that of the HA, the results rule out the possibility of significant contamination of the final products by any of the substances present during the reprecipitations. The finding of a loss of C^{14} greater than that of HA on reprecipitation is explicable on the basis of the presence of the label predominantly in oligosaccharides of lower molecular weight than the carrier HA. These oligosaccharides may remain partially in the supernatant fluid each time a reprecipitation is done.

TABLE 2

RECOVERY OF C^{14}-HYALURONIC ACID FOLLOWING REPRECIPITATION UNDER VARIOUS CONDITIONS

Exp. No.	Sp. Act. of HA Taken (Counts/ Min/ Mg GA)	Total HA Taken As GA (Mg)	Total HA Taken As C^{14} (Counts/ Min)	Calc. Dilution of C^{14} if HA Were Contaminated by C^{14}-labeled Substances Below: Mixture from Which HA Precipitated*		Calc. Dilution of C^{14} if HA Were Contaminated by C^{14}-labeled Substances Below: Mixture from Which HA Precipitated†		Recovery of HA Taken As GA (Per Cent)	Recovery of HA Taken As C^{14} (Per Cent)	Sp. Act. of HA (Counts/ Min/Mg GA)
6A	58.7	1.83	107	UDPAg	1.6×10^4	.....		79.8	35.3	25.9
				AG-6-P	1.9×10^2					
6A	58.7	1.83	107	.....		Acetate	6.2×10^3	95.1	38.1	23.4
						AG	1.6×10^3			
						Tetramer	3.8×10^2			
						Hexamer	1.9×10^2			
						Octamer	*ca.* 60			
6A	58.7	6.40	376	.....		Acetate	3.6×10^3	...	...	29.5
						AG	1.8×10^3			
						UDPAg	7.2×10			
						AG-6-P	3.6×10^2			
6A‡	29.5	0.81	24	.....		Tetramer	2.9×10^3	...	77.5	...
						Dimer	4.2×10^2			
6B	44.4	2.32	102	UDPAg	1.6×10^4	.....		89.6	70.2	34.5
				AG-6-P	7.1×10^4					
6C	47.5	2.18	103	AG-6-P	7.1×10^5	.....		76.1	42.3	26.2
8A	24.5	1.67	41	.....		H_2O only		105	50.0	11.6

* Precipitation made twice with 66 per cent ethanol and 0.3 per cent NaCl from neutral aqueous solution to give the calculated over-all dilution shown.

† Precipitation made once as above. "Octamer," "hexamer," "tetramer," and "dimer" refer to oligosaccharides present in a testicular hyaluronidase digest of HA.

‡ The HA taken for reprecipitation was that isolated in the presence of acetate, AG, UDPAg, and AG-6-P.

Experiment 8A of Table 2 shows that reprecipitation of the C^{14}-HA from water alone leads to a considerable loss of isotope with little loss of HA. The dialyzability of the C^{14}-labeled substances in the HA is in accord with these observations. Table 2 shows that the oligosaccharides synthesized must be larger than the octasaccharide, inasmuch as precipitation of the C^{14}-HA in the presence of large amounts of testicular hyaluronidase digest of unlabeled HA does not reduce the C^{14} content of the samples to any greater extent than any other kind of reprecipitation. The oligosaccharides in the hyaluronidase digest were chromatographed on paper in the butanol–acetic acid–water solvent (50:15:35) used by Meyer.[2] Their identity was established by their R_f values.

We have subjected one reprecipitated sample of C^{14}-HA to exhaustive digestion by testicular hyaluronidase, as shown in Table 3. The enzymatic digest was spotted multiply on a large sheet of Whatman No. 1 paper, together with several

spots of the undigested C^{14}-HA. After development with the butanol–acetic acid–water solvent, one strip of the undigested HA and one strip of the digested sample were sprayed with the Morgan and Elson type spray described by Partridge[23] to locate the regions of the oligosaccharides. There were no colored zones on the strip of the undigested HA. Regions corresponding to the disaccharide, tetrasaccharide, and hexasaccharide were found on the strip of the digested HA. Water eluates were made separately of the starting spots and of the lower areas of the sheet which corresponded to the location of the oligosaccharides as revealed by the sprayed strips. The eluates were plated and counted. Table 3 shows the results. In the case of the undigested HA, C^{14} was found only in the starting spot. Seventy per cent of the counts were recovered from this region. After hyaluronidase treatment, no C^{14} remained at the starting line; 58 per cent of the counts were recovered as the tetrasaccharide and 7 per cent as the disaccharide. Failure to recover all the radioactivity applied to the paper probably is due to the very small amounts of material present. The results of this experiment indicate that the oligosaccharides synthesized by the sarcoma homogenates have the structure of HA.

TABLE 3

PAPER-CHROMATOGRAPHIC ANALYSIS OF PRODUCTS FROM THE ACTION OF HYALURONIDASE ON C^{14}-HYALURONIC ACID*

			Recovery of C^{14} in Eluates of Areas of Paper Indicated						
			Starting Spot		Between Tetrasaccharide and Starting Spot	Tetrasaccharide		Disaccharide	
Material Spotted on Paper	Sp. Act. of HA or HA Digest (Counts/Min/Mg GA)	Total C^{14} Spotted (Counts/Min)	Total C^{14} (Counts/Min)	Sp. Act. (Counts/Min/Mg GA)	(Counts/Min)	Total C^{14} (Counts/Min)	Sp. Act. (Counts/Min/Mg GA)	Total C^{14} (Counts/Min)	Sp. Act. (Counts/Min/Mg GA)
Undigested C^{14}-HA	29.5	33.8	23.4	31.2	0	0	0	0	0
Digested C^{14}-HA	25.2	38.3	0	0	0	22.4	41.1	2.8†	36.4

* The C^{14}-HA was the once-reprecipitated 6A of Table 2 (line 3). 7.4 mg. of HA and 2 mg. of crystalline plasma albumin were dissolved in 1.5 ml. of 0.10 *M* acetate buffer, pH 4.7, containing 0.08 per cent NaCl. Testicular hyaluronidase (1,000 turbidity reducing units, Worthington Biochemical Corporation) was added. Incubated under toluene at 37° for 24 hrs. Solution heated at 100° for 10 minutes. Chromatogram prepared as described in text.

† The accuracy of this determination is low.

Some experiments have been done which shed light on the pathway of incorporation of C^{14}-AG-6-P into HA. The incorporation observed in the absence of added uridine nucleotides (experiment 6C, Table 1) can be abolished by pretreatment of the sarcoma homogenate at pH 8.0 and 0° with one-third of its volume of wet, thoroughly washed Dowex-1 (formate), 8 per cent cross-linked. The Dowex treatment was done twice with equal portions of the resin in bulk. Such treated homogenates incorporate C^{14}-AG-6-P in the presence of UDPG, DPN, and UTP but do not yield C^{14}-HA when UTP is omitted. When C^{14}-UDPAg is added as the only isotopically labeled substance, formation of C^{14}-HA occurs with the Dowex-treated homogenate to which UDPGA or UDPG and DPN as a source of UDPGA[16] have been added. Table 4 shows the results of several experiments of this kind. The homogenates do not appear to contain an active uridyl transferase which might mediate the following reaction:

$$\text{UDPG} + \text{AG-1-P} = \text{UDPAg} + \alpha\text{-Glucose-1-phosphate.} \qquad (2)$$

An enzyme of this type has been reported by Kalckar and coworkers to occur in extracts of *S. fragilis*, where uridine diphosphogalactose was found to be formed by a direct exchange between UDPG and α-galactose-1-phosphate.[24] Experiment 9C shows that the synthesis of HA does not occur directly from AG-1-P and, therefore, is not analogous to the synthesis of glycogen from α-glucose-1-phosphate. Experiment 9B shows that AG-1-P must first be "activated" by conversion to UDPAg according to equation (1) above, before the hexosamine unit can be incorporated enzymatically into HA. UDPAg was demonstrated chromatographically in the reaction mixture from experiment 9B. Experiment 9A, in which a large amount of unlabeled acetate was added as a "trap," shows that the incorporation of C^{14} into HA from UDPAg labeled with the isotope in the acetyl group does not proceed via acetate ion, despite the presence in the homogenates of an active deacetylase for AG-6-P or AG. Dorfman and coworkers have recently found that

TABLE 4

REQUIREMENT FOR URIDINE DIPHOSPHO-N-ACETYL-GLUCOSAMINE IN HYALURONIC ACID SYNTHESIS

Expt. No.	Treatment of Homogenate	Nucleotide Added (μmoles) UTP	UDPG	UDPGA	C^{14}-UDPAg*	C^{14}-AG-6-P* Added (μmoles)	C^{14}-HA Recovered (Mg.)†	Sp. Act. of C^{14}-HA (Counts/Min/Mg GA)	Reprecipitation of C^{14}-HA‡ HA Recovered (Per Cent)	C^{14} Recovered (Per Cent)
8A§	None	Trace	2.5	0.1	1.95	...	19.5	24.5	96	57
8C§	+Dowex-1	Trace	2.5	0.1	1.95	...	||	23.4	93	70
8D§	+Dowex-1	...	...	...	...	3.11	19.2	0.0	..	...
9A§	+Dowex-1	Trace	2.5	...	1.95	...	27.7	14.2	73	81
9B§	+Dowex-1	25.	2.5	...	...	3.11	24.8	10.6	90	38
9C§	+Dowex-1	...	2.5	...	...	3.11	25.6	3.7#	91	15#
9D§	+Dowex-1	...	...	...	...	3.11	29.4	3.1#	74	0

* Compound labeled with C^{14} in the carboxyl C of acetyl group. See Table 1.

† Total HA added to reaction mixture = 35.2 mg.

‡ In experiment 8, 3.2 mg., and in experiment 9, 5.5 mg., of C^{14}-HA reprecipitated with 66 per cent ethanol and 0.3 per cent NaCl in presence of 10 μmoles Na acetate, 0.2 μmole UDPAg, 2.3 μmoles AG, 0.58 μmole AG-6-P, 0.075 μmole N-acetyl-hyalobiuronic acid ("dimer"), and 0.52 μmole "tetramer."

§ Reaction mixture (7.8 ml.) contained: DPN, 2×10^{-3} *M*; $MgCl_2$, 4×10^{-3} *M*; THM, 8×10^{-3} *M*; EDTA, 4×10^{-4} *M*; glutathione, 10 mg. Enzyme: 4.5 ml. from 1.5 gm. tissue. 37°. 90 minutes. In experiments 8A and 8C, UDP, 6.4×10^{-4} *M*. In experiment 9A, Na acetate, 4.3×10^{-2} *M*. In experiments 9B, 9C, and 9D, α-glucose-1,6-diphosphate, 7×10^{-6} *M*. Experiment 8 maintained at pH 7.3–7.5. Experiment 9 maintained at pH 7.0–7.3.

|| No estimate available of total HA recovery since part of preparation was lost.

The accuracy of this determination is low.

the incorporation of AG labeled with C^{14} in the acetyl group into HA by whole cells of group A hemolytic streptococci could be attributed to its prior deacetylation to C^{14}-acetate.[25] This organism apparently possesses an active deacetylase for AG and was found to incorporate C^{14}-labeled glucosamine into HA. The authors discussed the possibility that HA synthesis proceeds from AG via acetate ion, which reacts with an unknown substance to form a precursor of the mucopolysaccharide. On the basis of what is now known about glucosamine metabolism, it seems likely that the unknown substance in their system was really Gm-6-P, which is acetylated by acetyl-coenzyme A, as has now been described.[11] The AG-6-P formed is incorporated into HA first by conversion to AG-1-P and then to UDPAg. It is known that Gm-6-P is formed by the action of yeast hexokinase on glucosamine.[26] The incorporation of C^{14}-glucosamine observed by Dorfman and coworkers was most likely due to prior phosphorylation of the free sugar by the hexokinase of the cells, one of the possibilities which they mentioned. Leloir and Cardini have reported that Gm-6-P is formed enzymatically in extracts of

Neurospora crassa by a kind of transamination from glutamine to hexose-6-phosphate.[19] We have found the N-acetylase for Gm-6-P to be active in the sarcoma homogenates. We have not yet tested the homogenates for the presence of the enzyme system described by Leloir and Cardini by which the Gm-6-P required for HA synthesis may be produced ultimately from blood glucose. Dorfman and coworkers have already shown that the carbon skeletons of the glucosamine and glucuronic acid units of streptococcal hyaluronic acid are derived from glucose without prior scission of the carbon chain.[27]

DISCUSSION

The work reported here shows that HA synthesis occurs enzymatically either by transfer of AG (and, presumably, of GA) residues from UDPAg (and UDPGA) to the terminal monosaccharide units of pre-existing chains or by preliminary reaction of these two uridine-linked sugars to form a uridine-linked disaccharide, which then adds its disaccharide unit to a pre-existing chain or to another uridine-linked oligosaccharide. Under the conditions we have studied, relatively short-chain oligosaccharides (M.W., $> 2{,}000$) apparently become the predominantly labeled species. The detailed mechanism and degree of reversibility of these synthetic reactions are being studied.

SUMMARY

1. Cell-free homogenates of the Rous chicken sarcoma have been found to synthesize oligosaccharide chains composed of N-acetyl-D-glucosamine and D-glucuronic acid units combined as in hyaluronic acid, by incubating them with C^{14}-N-acetyl-D-glucosamine-6-phosphate and uridine triphosphate or with C^{14}-uridine diphospho-N-acetyl-D-glucosamine. In either case, the source of glucuronic acid units was presumably uridine diphospho-D–glucuronic acid, formed by enzymatic oxidation of added uridine diphosphoglucose in the presence of diphosphopyridine nucleotide.

2. The C^{14} label has been shown to be a part of the oligosaccharide structure by reisolation of the C^{14}-hyaluronic acid from large molar excesses of all substances, added in nonisotopic form, which might have been present as C^{14}-labeled contaminants in it.

3. Presence of the C^{14} label in the oligosaccharides themselves has also been shown by exhaustive enzymatic digestion of the C^{14}-hyaluronic acid with testicular hyaluronidase and subsequent chromatographic isolation of a small amount of C^{14}-N-acetyl–hyalobiuronic acid and of a large amount of the corresponding C^{14}-labeled tetrasaccharide.

4. The incorporation of isotope from C^{14}-N-acetyl-D-glucosamine-6-phosphate has been shown to occur via uridine diphospho-N-acetyl-glucosamine, which appears to be an obligatory intermediate.

5. The sarcoma homogenates have been shown to contain all the enzymes necessary to synthesize hyaluronic acid from uridine diphosphoglucose and D-glucosamine-6-phosphate. The pathway of this synthesis is discussed.

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[4] The symbols used in the text refer to compounds as follows: AG, N-acetyl-D-glucosamine; AG-6-P, N-acetyl-glucosamine-6-phosphate; AG-1-P, N-acetylglucosamine-1-phosphate; Gm-6-P, D-glucosamine-6-phosphate; GA, D-glucuronic acid; UDPAg, uridine diphospho-N-acetyl-glucosamine; UPDGA, uridine diphosphoglucuronic acid; UDPG, uridine diphosphoglucose; UTP, uridine triphosphate; UDP, uridine diphosphate; UMP-5′, uridine-5′-phosphate; HA, hyaluronic acid; DPN, diphosphopyridine nucleotide; EDTA, ethylenediamine tetraacetic acid; THM, tris-(hydroxymethyl)-aminomethane.

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*ELECTRON-MICROSCOPIC EVIDENCE FOR THE LOCALIZATION OF RIBONUCLEIC ACID IN THE PARTICLES OF TOBACCO MOSAIC VIRUS**

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Many recent physical investigations of purified tobacco mosaic virus have yielded an essentially consistent picture of the virus particles. Electron microscopy[1] has indicated that the particles are highly uniform rods measuring 3,000 × 150 A. From a correlation of particle counts with mass concentration for a virus suspension,[2] the mass per unit particle has been found to be 8.2×10^{-17} gm., or about 50×10^6 atomic mass units. Ultracentrifuge studies have also indicated a high uniformity in the material[3] and have further shown that the basic infective unit sediments with the predominant particle,[4] whose mass and dimensions are given above. However, the cross-sectional shape of the virus rod is in question. High-resolution microscopy has offered some evidence of a hexagonal cross-section,[5] but X-ray diffraction studies[6] indicate that the particle has a helical arrangement of diffracting groups, a helix in which there are a nonintegral number of equivalent, or nearly equivalent, diffracting groups per turn. The pitch is about 22 A, and there is some evidence of an external grooving of the rod between adjacent turns.[7] However, no interpretable surface detail has been revealed by electron microscopy, a result which is not surprising, since the limit of resolution for present-day instruments is quite close to the 22 A distance given for the helical pitch.

Although the virus is known to be a nucleoprotein, little is known about its internal structure. Cohen and Stanley[8] have found that by heat denaturation in salt solution, followed by centrifugation, it can be split into fractions of protein and ribonucleic acid (RNA), which constitute, respectively, 94 per cent and 6 per cent of the mass. The ease of separation suggests an arrangement of the two kinds of material into separate substructures in the particle. In one such arrangement, proposed by Watson,[6] the RNA forms a central core running the length of the rod, surrounded by a superhelix of protein. He based that proposal on the observation that in T2 bacteriophage, and apparently in turnip yellow mosaic virus —two widely different viruses—the nucleic acid component is surrounded by protein. The purpose of the present investigation was to obtain electron-microscopic evidence for or against the existence of such a structure in tobacco mosaic virus.

Results and Discussion.—Two kinds of evidence bearing on the location of the RNA in tobacco mosaic virus have been found. The first was obtained by examining partially degraded virus particles. After trial of a number of methods of degradation, one was found which gave a systematic and controllable breakdown of the virus rods and yielded material suitable for electron microscopy. The method was as follows: The purified virus at a concentration of 0.05 per cent was heated for various periods at 85° C. in a 0.02 per cent solution of Duponol C, an anionic detergent. The solution was then cooled and diluted about thirty fold in water for spraying[9] upon microscope grids. Figure 1 is a micrograph of material heat-treated for 60 seconds. The rods are of the same apparent width as untreated particles but somewhat shorter. Attached more or less centrally to the ends of the

rods are fibers, which, according to the shadow widths,[10] are one-fourth to one-fifth the thickness of the rods, or between 30 and 40 A in diameter. Continued heating of the Duponol-virus mixture resulted in a shortening and finally a disappearance of the rods, and in the appearance of naked fibers, many of which were about 3,000 A long.

Figure 2 shows material which was heat-treated for the same length of time but was then diluted in a 0.00001 per cent solution of crystalline ribonuclease and incubated for an hour at 37° C. before spraying. Here the fibers have almost entirely disappeared. In control tests where crystalline trypsin was substituted for ribonuclease at fifty times the concentration at which the latter had been used, the fibers were not removed. Thus there appears to be an RNA-containing fiber which runs parallel to the rod axis and which is less readily "dissolved" than the rest of the rod in hot detergent.

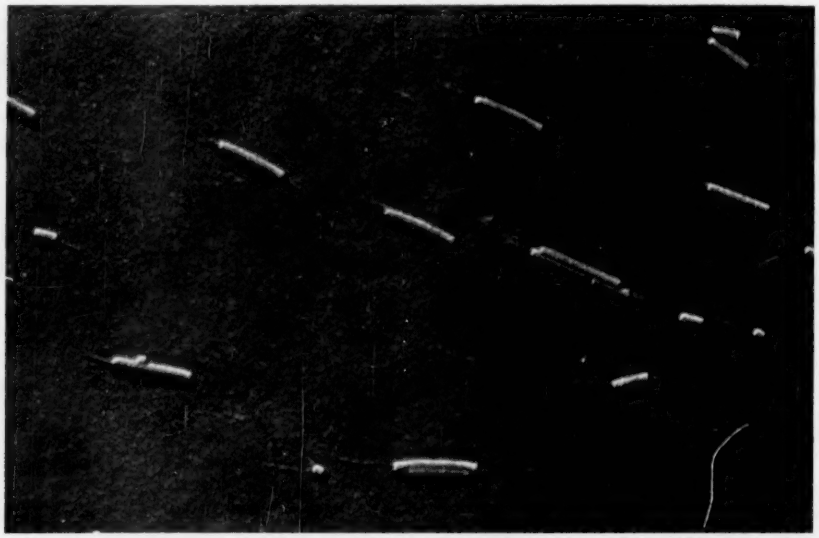

FIG. 1.—Tobacco mosaic virus particles after treatment with a hot detergent solution. The narrow fibers seen projecting from the ends of the rods consist of ribonucleic acid, which presumably is localized along the axis of the virus rod. × 60,000.

The second line of evidence comes from an examination of X-protein,[11] a substance found with tobacco mosaic virus in infected plants. X-protein is immunologically related to the virus, but it differs in electrophoretic mobility; it is non-infective, and it is free of nucleic acid. The state of aggregation of the protein varies reversibly with hydrogen-ion concentration: at pH 5 the material is in the form of rods of the same apparent thickness as the virus and of various lengths, some as great as 3,000 A. Figure 3 shows a sample of X-protein. The field contains several particles which, as evidenced by their shadows, are short rods that happened to be left standing on end when the spray droplet dried. In such an end-on view, the presence of a pit or hole at the center is clearly shown. Thus a tubular structure is indicated for X-protein. The width of the central channel is apparently between 30 and 70 A. It should be noted that such a hole or channel was not

observed in corresponding views of short rod fragments obtained by sonication of the whole virus.[5]

If it is assumed that the protein in the virus is aggregated in the same manner as X-protein, then the central channel is the only space available for an RNA fiber as large as those shown in Figure 1. Thus the two lines of evidence indicate that the

FIG. 2.—Tobacco mosaic virus particles treated with detergent as in Fig. 1 but subsequently incubated in a ribonuclease solution. The fibers seen in Fig. 1 have been almost entirely removed by the action of the enzyme. × 60,000.

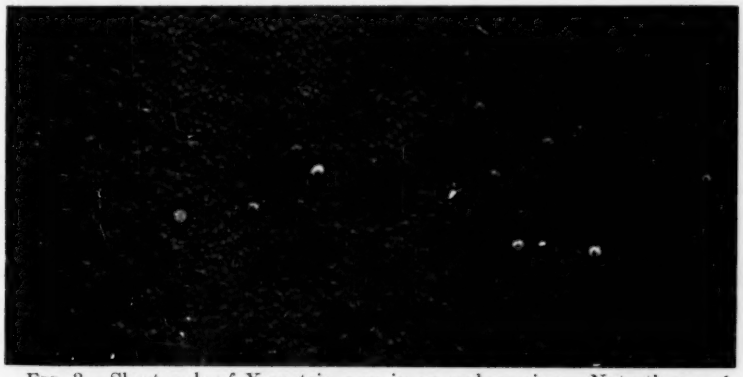

FIG. 3.—Short rods of X-protein seen in an end-on view. Note that each rod appears to have a depression or pit at its axis. × 80,000.

RNA forms an axial core, as proposed by Watson. Further, the core diameter which can be calculated from the known amount of RNA per particle[6] is in agreement with the width of the observed fibers.

Summary.—The ribonucleic acid in tobacco mosaic virus appears to be localized in a central core running the length of the rod-shaped virus particle. This conclusion is based on (*a*) observation of partially degraded particles from which project fibers some 30–40 A in diameter; (*b*) lability of the fibers in trace concentrations

of ribonuclease; (*c*) observation of a hole or channel at the center of short rods of X-protein, which contains no nucleic acid.

The author is greatly indebted to Professor Robley C. Williams for instruction and for constant help, advice, and encouragement. He also wishes to thank Professor C. Arthur Knight for samples of tobacco mosaic virus and Professor William N. Takahashi for his sample of X-protein.

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*NONVISUAL FUNCTIONING OF OCCIPITAL CORTEX IN THE MONKEY**

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In neurological theory the cerebral cortex has come to be regarded as a mosaic of specialized anatomico-functional regions. Recent work on the behavioral effects of cortical lesions in the monkey has, in general, supported this view.[1] Striate cortex is the example par excellence of a region highly differentiated in a structural and functional sense.[2] Its boundaries can be determined with the unaided eye, and its participation in vision is unquestioned.

It has been proposed a number of times that regions of the cortex participate in more general nonspecific functions as well. Over a century ago Flourens proposed an *action commune* in addition to the *action propre* of each part of the brain.[3] Since then, Goltz[4] and Lashley[5] especially have propounded similar notions. Lashley demonstrated experimentally the nonspecialized functioning of virtually every sensorimotor zone in the cortex of the rat.[6] Maze performance of cortically blinded rats, for example, was extremely inefficient as compared with normals *or with peripherally blinded rats*. The same effect was demonstrated in rats enucleated just after birth to rule out the possibility that learned imaginal processes were disrupted by the cortical lesions.[7] Clearly, occipital tissue was involved in the nonvisual solution of the maze problem and in the maintenace of efficient performance. Lashley argued that the principle of localization be re-examined and proposed the theory of mass action to account for data such as these.

Recently the present writer launched an experimental program to compare the effects of brain lesions in the monkey with those reported by Lashley and others in the rat.[5] Homologies in cortical regions of the two species are often difficult to draw, but striate cortex is exceptional in this respect and was chosen for initial study. The immediate aim was to demonstrate that striate cortex participates in nonspecific, nonvisual problem-solving and retention in the monkey. Tasks, too, are difficult to "homologize." Initially, a large battery of nonvisual tests was constructed, including simple discriminations (somesthetic and auditory), tests of generalization and transfer (in somesthesis), and tests of ability to respond differentially according to context (delayed response, alternation, and conditional reaction). A stylus maze, duplicating the pattern of the locomotor maze used by Lashley (maze 3),[6] was also included in the battery. Four rhesus monkeys learned these tasks in total darkness to insure that response would be controlled by nonvisual cues. Observation was possible with the use of a snooperscope, a device which converts infrared light to visible light seen by the experimenter only. Preoperative retention scores were obtained and served as a base line to which postoperative retention scores were compared.

It is enormously difficult to remove striate cortex without damage to other structures in a fissurated brain like the macaque's, where a third or more is hidden in the depths of the calcarine fissure. In contrast to the case of the rat, however, there is no danger of damaging subcortical nuclei. In order to spare nonstriate cortex, partial striate cortex lesions were produced in two monkeys. In the remaining two, virtually all striate cortex was removed, with concomitant damage to peristriate cortex on the medial surface of the hemispheres. Thalamic studies of retrograde degeneration indicated that less than 1.3 per cent of the neurons remained in the lateral geniculate bodies. All operations were performed in one stage, using aseptic precautions. Tissue was removed by blunt dissection and aspiration.

The operation appeared to have no effect on the nonvisual behavior of any of the monkeys.[8] The delayed-response performance suffered somewhat in one monkey but was unaffected in the remaining three. It is especially to be noted that the stylus maze habit was extremely well retained by all four. These monkeys were unquestionably efficient performers on all the tasks outlined above. Clearly, no support for Lashley's contention that striate cortex exerts a facilitative action on other regions of the brain was indicated.

In an attempt to rule out the remaining doubt in the writer's mind, a locomotor replica of Lashley's maze 3, suitable for monkeys, was constructed. Five naïve rhesus monkeys and one sophisticated one were selected as subjects. The procedure followed Lashley's in every detail.[9] The monkeys learned to traverse the maze errorlessly, after which they were peripherally blinded. They were enormously affected by the lack of vision but eventually reached the same pre-established level of accuracy. Following a two-week rest interval, retention for the habit was determined (again used as base line to which postoperative retention was contrasted). Large occipital lesions were produced, involving virtually all striate cortex and a sizable portion of peristriate cortex. A two-stage operation was performed on three monkeys, a one-stage operation in the remaining three. Testing commenced two weeks after operation in each case.

The unilateral lesions appeared to have no effect on the maze habit (one of these three monkeys succumbed to a severe attack of dysentery before the second operation). On the other hand, bilateral occipital lesions (produced in one or two stages) had a consistently adverse effect on maze performance. All monkeys made a larger number of errors and traversed the maze more slowly after both hemispheres were involved in the lesion. Total errors made in the first ten trials of testing are summarized in Table 1. Deficits were in no way as striking as those re-

TABLE 1

ERRORS MADE DURING THE FIRST TEN TRIALS OF TESTING IN TRAVERSING AN EIGHT CUL-DE-SAC MAZE

	SUBJECTS					
	Styx	9	10	11	12	13
After two-week rest preoperatively	1	12	4	6	..	..
After unilateral lesion	2	..	..	..	11	6
After bilateral lesion	603	26	41	10	44	..

ported for the rat, but the maze performance, though mildly inefficient, was in marked contrast to the lack of effect on the host of nonvisual tasks and the stylus maze used previously. Additional confirmation of these facts was provided by one of the maze-deteriorated monkeys who learned and was tested on a number of the nonvisual discrimination tasks, none of which suffered as a result of the operation.

In summary, the data indicate the following: (1) Nonvisual discriminations of varying degrees of complexity and stylus maze performance are not affected by occipital lesions. (2) Locomotor maze performance suffers as a result of occipital lesions. The monkeys do not appear "demented,"[7] nor have they completely lost the habit,[9] since some show large savings in relearning the maze. This result is to be contrasted, on the one hand, with the more deteriorated maze performance reported for the rat and, on the other, with the lack of effects on discrimination tasks of varying complexity in the monkey. Both one- and two-stage operations lead to the same effects. And problem-solving sophistication seems not to be a relevant factor. (3) The differential effects summarized in points 1 and 2 have been recorded in a single monkey.

No marked success has yet been achieved in an analysis of the differential requirements for the solution of locomotor and stylus maze problems.

There is no way of ruling out at present the possibility that the locomotor maze deficits are related to the large peristriate cortex lesions and not at all (or only partly) to the "primary visual" cortex. If this should eventually prove to be the case, it will provide additional evidence opposing the traditional view that peristriate cortex constitutes a visual "association" area in the monkey.

The principle of corticalization of function has received much support from comparative studies of the visual system. It is fitting, therefore, that striate cortex should provide evidence of lack of complete specificity of function. From a comparative point of view, the present data, together with Lashley's and Tsang's, suggest that increasing specificity proceeds in parallel with increasing corticalization as the phylogenetic scale is ascended. And, just as corticalization is incomplete in the monkey, so is specificity of cortical function.

* A detailed report of the data and anatomical verification of the lesions will appear elsewhere.

† The author acknowledges the advice and encouragement of Dr. K. S. Lashley.

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DO FUCUS EGGS INTERACT THROUGH A CO_2-pH GRADIENT?

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In 1889 Rosenvinge[1] reported that the direction of the main morphological axis, or polarity, of the eggs of the Fucaceae could be determined by neighboring eggs. Developing in darkness, they were found to germinate proximally, i.e., to develop their rhizoids or basal poles toward their neighbors. This so-called "positive group effect" has been well confirmed,[2, 3, 4] though a tendency toward proximal germination between the smallest of groups, a pair of eggs, was found to occur only below pH 7.6.[5, 6]

An understanding of this mutual orientation would bear upon two broader problems: the origin of polarity and the mechanisms of morphogenetic interaction between cells. Rosenvinge[1] suggested an oxygen gradient to be responsible for the positive group effect; Olsen and du Buy,[7] an auxin gradient; Whitaker,[8] a CO_2-pH gradient. Whitaker supported his proposal by demonstrating that when *Fucus* eggs are exposed to a CO_2-pH gradient in darkness, where they respire, they do, in fact, germinate toward the high CO_2, high H^+ pole (except at very low pH's, on the border line of growth inhibition).[9] Moreover, so many other facts fit neatly into this picture that Whitaker's hypothesis has won wide acceptance: In a gradient of white light, rhizoids develop from the darker side; in a thermal gradient, from the warmer side; in a gradient of dinitrophenol (averaging 10^{-5} M at threshold) or of potassium indole acetate (averaging 10^{-3} M at threshold), from the side of higher concentration.[8, 10]

However, the positive group effect has been reported to appear in illuminated, and hence presumably photosynthesizing, cultures.[1, 2, 3] Although this suggests oriented development in a direction opposite that expected on the basis of Whitaker's hypothesis, the results are ambiguous. First, it cannot be inferred from the published data that the illuminated eggs maintained net photosynthesis. Second, the observed effects may in part have been due to mutual shading of the eggs, since in a gradient of white light rhizoids develop from the darker sides. For these

reasons the author has conducted further experiments on the germination of *Fucus* eggs, illuminated under conditions not subject to the above criticism.

Light gradients were minimized by illuminating the cultures directly from above and, by means of mirrors, also from below, so that very little light struck the eggs at angles low enough to cause shadowing. Furthermore, red instead of white light was used, because red light has been reported not to orient *Fucus* eggs even when unilaterally applied.[3, 11] This claim has been verified in experiments with eggs grown under conditions identical with those used to study the mutual orientation of illuminated pairs, except that the light was directed unilaterally instead of from above and below. The results showed that 57 per cent of 222 eggs had germinated from their darker hemispheres and 43 per cent from their lighter ones.

The red light used in all experiments was obtained by filtering light issuing from a tungsten filament lamp through $^1/_2$ meter of water and an F-29 Kodak Wratten filter reported to eliminate radiation of wave lengths shorter than 600 mμ; its intensity was *ca.* 800 foot-candles.

The experiments were conducted with zygotes of *F. furcatus*, obtained as described by Whitaker,[12] and were carried out in an 11° ± $^1/_2$° C. constant-temperature room or water bath.

1. A suspension of recently fertilized *Fucus* eggs in filtered sea water was placed in a cylindrical culture chamber. After settling, the eggs nearly covered the bottom with a single layer of cells. The chamber was closed with a cover slip so as to exclude gas bubbles, sealed with paraffin oil, and illuminated with red light.

In this culture gas bubbles accumulated; after 21 and 33 hours their estimated volumes were 5 and 9 μl., respectively. No gas bubbles were observed in a control experiment with sea water without eggs and sterilized with $HgCl_2$. The culture was then left in darkness for 36 hours; during this period the gas volume fell to 1 μl.

Examination of the culture after 33 hours of illumination showed that practically 100 per cent of the eggs bordering the few clear spaces had germinated away from these spaces and toward their neighbors. If the gas production is attributed to O_2 evolution by photosynthesis, this simple experiment shows that the positive group effect is not mediated by a CO_2 (or an O_2) gradient.

2. This was corroborated by an experiment with more widely spaced eggs. That net photosynthesis was maintained here was demonstrated in a parallel experiment using Warburg manometers.

Three thousand recently fertilized eggs were distributed on the bottom of a Carrel flask (5 cm. in diameter) containing 8 cc. of sea water buffered at pH 6.0 with 0.016 *M* phosphate, plus 2 cc. of natural sea water added just before the flask was sealed so as to provide sufficient CO_2 for prolonged photosynthesis (5×10^{-4} *M* $CO_2 + HCO_3^-$). The flask was illuminated with red light until after germination was completed. Then the culture was carefully scanned for semi-isolated pairs of eggs (those less than $1^1/_2$ egg diameters apart whose separation from the nearest third egg was at least 3 times their distance apart). Of 78 eggs observed in such illuminated pairs, 82 per cent had germinated proximally, the rest distally. Two control cultures developed in darkness. In one, bearing an initially identical medium, 68 per cent of 84 eggs germinated proximally. In another dark control, not supplemented with natural sea water, 77 per cent of 88 eggs germinated proximally. Thus, if anything, the illuminated, photosynthesizing culture showed a more marked positive group effect than the dark, respiring controls.

3. In the parallel manometric experiment, four test vessels plus two thermobarometers were so rigged as to allow determinations of both oxygen and carbon dioxide exchange, in darkness as well as in red light. Each vessel contained in its main compartment 2 ml. of artificial sea water, buffered at pH 6.0 with 0.015 *M* phosphate; the four test vessels were each supplied with 17 mm.^{3} of eggs. Two of the test vessels were illuminated with red light, while two were kept in darkness. One illuminated test vessel, one "dark" vessel, and one thermobarometer each contained in its center well and appendices 1 ml. of a CO_2 buffer to maintain a constant pressure of 0.3 per cent CO_2 in the gas phase (0.321 *M* $KHCO_3$ plus 0.154 *M* Na_2CO_3).[13] Here the pressure changes were due exclusively to oxygen production or utilization. Carbon dioxide assimilation or production were computed from simultaneously measured pressure changes in the companion vessels without CO_2 buffer, equilibrated with a gas phase composed of air plus 0.3 per cent CO_2; any decrease in the rate of photosynthesis resulting from CO_2 depletion was minimized by flushing the illuminated vessel at approximately 3-hour intervals with this gas mixture. The experiment was conducted in a water bath at 11° C.; photosynthesis was measured over the period from $2^1/_2$ to 23 hours after fertilization; nearly all the eggs had germinated. Measurements in the dark were continued for another 13 hours, at which time germination had here been practically completed.

The results show that the rate of photosynthesis, not corrected for respiration, remained constant at 0.8 μl gas/mm^3 eggs × hr, double the rate of respiration, which was constant at 0.4 μl gas/mm^3 eggs × hr. Both the assimilatory and respiratory quotients were within 5 per cent of unity.

In so far as they are comparable, these results harmonize with those of Whitaker,[14] who found that at 18° C. fertilized *F. vesiculosus* eggs produced more than 2 μl O_2/mm^3 eggs × hr in white light at an intensity of 100,000 foot-candles and consumed 1.0 μl O_2/mm^3 eggs × hr in darkness.

These experiments convincingly demonstrate that in red light photosynthesis exceeded respiration, and calculations readily provide assurance that, in the stationary cultures used to study the group effect, diffusion brought enough CO_2 to the eggs to maintain this condition without the 24-hour period needed to complete germination. Hence it can be safely inferred that the positive group effect persisted in red light, despite the fact that the CO_2-pH gradient was opposite that of the dark cultures.

4. Further evidence against the hypothesis that a CO_2-pH gradient is responsible for proximal germination is furnished by the failure of wide variations in buffer capacity to influence the group effect. Since orientation is not an all-or-none phenomenon, and its manifestation seems to depend on the steepness of a gradient, it should be expected that, the greater the latter, the more pronounced the degree of orientation.

The author has examined shadowgraphs of cultures of eggs, in sea water buffered at pH 6.0 with 0.016 and 0.002 *M* phosphate, respectively, after development in darkness. In each experiment 3,000 eggs were distributed over the bottom of Petri dishes 5 cm. in diameter. The shadowgraphs were analyzed so as to allow direct comparison with the results of Whitaker's[5] similar exeriments on dark-grown *F. furcatus* eggs in sea water buffered at pH 6.0 either with 0.008 *M* phosphate-citrate or with bicarbonate; in the latter case the medium was in atmospheric equilibrium and hence contained 0.000024 *M* CO_2 plus HCO_3^-. The concentration near a pair

of eggs must have been slightly greater due to respiration; it can be estimated as 0.00003 M.

Mutual orientation was determined for egg pairs separated from each other by $^1/_2$–$1^1/_2$ egg diameters, and more than 7 egg diameters from the nearest third egg. The extent of orientation has been listed in Table 1; it is expressed under two categories, viz., general orientation, covering the cases of rhizoids developing proximally, 0°–90° toward their neighbors, and strong orientation, for rhizoid development in the direction of neighboring cells within 10° of the line joining the egg centers.

TABLE 1

MUTUAL ORIENTATIONS OF PAIRS OF EGGS REARED IN SEA WATER AT pH 6.0 IN VARIOUS BUFFERS

OBSERVER	BUFFER	TOTAL NO. OF EGGS	PER CENT OF ALL RHIZOIDS		
			Distal 90°–180°	Proximal 0°–90°	Proximal 0°–10°
W	3×10^{-5} M carbonate	104	12	88	26
J	2×10^{-3} M phosphate	74	11	89	23
W	8×10^{-3} M phosphate + citrate	97	8	92	33
J	1.6×10^{-2} M phosphate	48	11	89	29

It is clear that the results of Whitaker's experiments and mine are fully comparable; over the 500-fold range of buffer concentrations involved, no difference is observed. Since the first pK of carbonic acid and the second of phosphoric acid in sea water are both about 6.1, the range of buffer concentrations also covers an equal range of buffer capacities. And, because the steepnesses of the H^+ ion gradients established are inversely proportional to the buffer capacities,[15] it may therefore be concluded that the identical orientations cannot be attributed to these gradients.

Summary.—The tendency of *Fucus* eggs to develop their basal poles toward each other persists despite the replacement of respiratory CO_2 emission by photosynthetic CO_2 uptake and is not influenced by a 500-fold change in buffer capacity. Hence this interaction is not mediated by gradients of H^+, CO_2, or O_2.

* National Science Foundation Postdoctoral Fellow. Most of this work was done while the author held a National Research Council Postdoctoral Fellowship.

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RESISTANCE AND SUSCEPTIBILITY OF CARROT ROOTS TO CROWN-GALL TUMOR FORMATION

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Crown-gall tumor cells induced by *Agrobacterium tumefaciens* (Sm. and Town.) Conn develop from normal plant cells by the action on them, in sequence, of several causal factors.[1] Inoculations of susceptible species frequently result in fairly uniform tumors in 100 per cent of the trials. Inoculations of other plant species are not always successful or may result in tumors showing considerable size variations. Smith *et al.*[2] and Magnus[3] noted that different carrots formed tumors showing a gradient in size. DeRopp,[4] in this laboratory, found that carrot roots could be divided into three groups relative to the amount of tumor tissue formed on transverse or longitudinal slabs of carrot root. He termed these "strong," "moderate," or "nonreactor" carrots, depending on whether the inoculated surface formed large, coalescing masses of tumor tissue, a moderate number of discrete or barely confluent tumors, or no tumor tissues. These reaction categories were independent of the strain of bacteria and were not ascribable to varietal differences or to growth conditions.

During the development of a quantitative bio-assay for tumor formation,[5] it was found that disks of young secondary phloem excised from radial cylinders of different carrot roots and inoculated on their cambial-adjacent face exhibited similar variations in tumor-forming capacity. The weight of tumor tissue per disk in 15 days ranged from less than 5 mg. to over 200 mg. Phloem tissue possessing any predetermined tumor-forming capacity continued to show this capacity within 10–15 per cent even after two to three months storage at 8° C. The differences in tumor formation between the strong reactor (SR) and the weak reactor (WR) carrots might be ascribed to the presence of an inhibitor in the WR carrots or to the absence from them of one or more substances necessary for optimal tumor formation. This report is concerned with an investigation of these alternatives.

Methods.—Disks of young secondary phloem were cut from radial cylinders of carrot roots of predetermined tumor-forming capacity. WR disks were taken from roots which formed less than 20 mg. of tumor tissue in 15 days, and SR disks were excised from carrots forming more than 80 mg. of tumor tissue under the same conditions. *Agrobacterium tumefaciens*, strain B6, used throughout this study, was grown in nutrient broth in shake cultures for 18 hours at 26° C. A 5-mm. platinum loop delivered *ca.* 10^6 bacteria to each disk, and the suspension was spread over the cambial-adjacent surface of the disk. Carrot juice (wound juice) was prepared by grinding peeled carrots in a meat-chopper, pressing out the juice in a Carver press, clarifying for 30 minutes at 10,000 × g, and sterilizing by successive passage through 015, 02, and 03 Selas porcelain filter candles. Eight to 12 disks were used per variable in each experiment. All were incubated at 26° C. on moistened filter paper in Petri dishes.

Results.—Disks of phloem tissue from known SR and WR carrots were placed in Petri dishes, and 0.1 ml. of wound juice from either SR or WR carrots was ap-

plied to their cambial-adjacent surfaces. At the same time, healed disks, those cut from carrots one week before the experiment and allowed to heal in Petri dishes, were similarly treated. After the wound juice had been allowed to soak into the tissues for two to three hours, the disks were inoculated. The juice from SR carrots, although without effect on tumor formation on SR disks, shortened the time of tumor appearance and increased the final tumor weight on disks of WR phloem (Table 1). Juice from WR carrots did not affect tumor formation on SR disks and was also without effect on WR disks. Wound juice from SR carrots also induced more tumor formation on healed disks than did WR juice. It was concluded that the resistance of WR carrots to tumor production is due to the absence from their cells of substances necessary for tumor formation and not to an inhibitor.

The wound-juice factor is not specific. It is absent or inactive in canned carrot juice and in fresh or canned tomato juice. It is present in the wound juices prepared from the storage organs of several related or unrelated plants (Table 2). Its apparent absence from the wound juice of other plants (*Geranium* and *Bryophyllum* stems) appears to be due to the presence of interfering compounds which suppress tumor formation on both SR and WR carrot disks.

TABLE 1

EFFECT OF WOUND JUICE FROM KNOWN STRONG AND WEAK REACTOR CARROTS ON TUMOR FORMATION ON FRESHLY CUT AND HEALED DISKS OF CAMBIAL-ADJACENT SECONDARY PHLOEM FROM STRONG OR WEAK REACTOR CARROTS

	Strong Reactor Disks						Weak Reactor Disks					
	Control		Strong Juice		Weak Juice		Control		Strong Juice		Weak Juice	
	Days*	Mg.	Days	Mg.	Days	Mg.	Days	Mg.	Days	Mg.	Days	Mg.
Fresh-cut disks	6	89	6	95	6	93	7	10	6	57	7	10
Healed disks	...	0	7	42	8	22	...	0	8	45	9	9

* Days to macroscopic tumor formation.

TABLE 2

EFFECT OF WOUND JUICE FROM VARIOUS PLANTS ON TUMOR FORMATION ON DISKS OF FRESHLY CUT PHLOEM TISSUES FROM STRONG AND WEAK REACTOR CARROT ROOTS

	Mg. Tumor Tissue/Disk	
Wound Juice	Strong Reactor Disks	Weak Reactor Disks
None	95	23
Carrot root	95	55
Turnip root	100	70
Beet root	94	50
Parsnip root	96	78
Potato tuber	92	42
Geranium stem	50	0
Bryophyllum stem	60	0

The juice factor may be diluted out with water, and at dilutions below 50 per cent the juice factor from SR carrots had little or no effect on tumor formation by WR tissues. It is stable to freezing at $-10°$ C., to temperatures of 100° C. for 5–10 minutes, and to lyophilization. It does not dialyze into 10^{-3} *M* phosphate buffer, pH 6.8, and is insoluble in, and may be precipitated from, 80 per cent chilled acetone. It is not precipitated when the wound juice is saturated with ammonium sulfate at 4° C. or at pH 2.0, a pH at which the proteins of carrot juice are denatured. These characteristics suggest that the wound-juice factor may be a large, nonproteinaceous molecule.

The failure of other tissues of the carrot root to form large tumors[5] was also investigated. The cambial-adjacent secondary xylem, which may be presumed to be composed of "young cells", formed small tumors. However, xylem tissues of SR roots possess the wound-juice factor required for tumor formation on WR phloem disks and do not contain an inhibitor for tumor formation on SR phloem disks. Xylem tissues, stimulated into callus-cell formation by 10^{-7} gm./l. naphthaleneacetic acid, were inoculated by scoring with a scalpel dipped into crown-gall bacteria. No significant increase in tumor formation was noted on these disks as compared with appropriate controls. It was concluded that xylem cells of carrot roots are relatively refractory to tumor formation, independent of the wound-juice factor and of their stage of the growth activity.

Older secondary phloem of SR roots, that adjacent to the pericycle and periderm, was also refractory to tumor formation. In these older phloem tissues an inhibitor was found which masked the wound-juice factor. Reciprocal juice experiments showed that this inhibitor suppressed tumor formation on cambial-adjacent phloem tissues from the same root. This inhibitor was labile at 100° C. within 5 minutes when WR phloem disks were used as the assay system. It was antagonized to some extent by the wound-juice factor from young phloem.

Discussion and Conclusions.—The role of the juice factor in the formation of crown-gall tumor cells is, at present, not clearly understood. Examination of the tumors formed on carrot tissues possessing different grades of tumor-forming capacity showed that tumors of weak reactor phloem disks are both small in size and few in number. When weak reactor disks were supplemented with wound juice from SR carrots, the resulting tumors not only were more numerous but individually attained greater sizes than did those on control disks. Thus the juice factor acts in both a quantitative and a qualitative manner. A quantitative role for the juice factor is readily understood: to induce many tumors per disk, sufficient juice factor is necessary. The qualitative role may have the same basis, sufficient juice factor being necessary for more than minimal transformation of normal cells into tumor cells, minimal transformation resulting in the formation of primary tumor cells whose progeny form slow-growing tumors. The juice factor has no effect on the course of tumor development on either SR or WR disks after the period of the alteration of normal into tumor cells. Tumor development was not affected by growing inoculated disks on White's nutrient or on water agar. It is concluded that the juice factor plays a role in the initiation of tumor cells but is not required during tumor-cell growth and duplication.

Summary.—Crown-gall tumor-cell formation on standardized disks of the cambial-adjacent phloem of carrot root is, in part, a function of a factor(s) present in the cells of these and other tissues of the root. Disks of phloem tissue from carrots that react strongly to tumor formation are such because they contain in their cells a factor necessary for tumor-cell formation which is made available for this process when the tissues are wounded. Those carrots which react slightly, if at all, apparently contain little or none of this factor, and those carrots that form intermediate amounts of tumor tissue appear to contain moderate amounts of the wound-juice factor. The nature and the roles of the wound-juice factor in tumor-cell formation are now under investigation.

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CONDUCTANCE OF STRONG ELECTROLYTES AT FINITE DILUTIONS

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The equivalent conductance Λ of strong electrolytes in solvents of high dielectric constant approaches linearity in the square root of concentration in the limit of infinite dilution. At finite dilutions, the conductance curve is concave upwards. The slope of the limiting tangent has been computed theoretically by Onsager[1, 2] in terms of ionic charge, dielectric constant D and viscosity η of the solvent, absolute temperature T, universal constants, and one arbitrary constant, the limiting conductance Λ_0 of the solute. It has been surmised that the course of the actual conductance curve is a consequence of the finite size of real ions and of terms higher than linear in the charge of the reference ion; these were neglected in the derivation of the limiting law. It is the purpose of this communication to derive a conductance equation which is valid for nonzero concentrations. One additional arbitrary constant, the ion size a, suffices to reproduce experimental data up to about 0.1 normal for strong 1-1 electrolytes in water.

Briefly summarized, the derivation proceeds as follows: We start with the general equation of continuity[3] which specifies the concentration of ions of one species in the vicinity of ions of other species in a solution of electrolyte which has reached a steady state under the influence of an external force (here, an electric field). The equation is first solved, neglecting terms of higher order, but using ions of finite size rather than point charges as the model. This first-order solution is then returned to the higher-order terms in the equation of continuity. Solution of the resulting perturbation problem gives the desired expression for the relaxation effect in the mobility to terms of order c and $c \log c$. On combining this result with the previous value[3] of the electrophoresis calculated to the same order, the final conductance function is obtained.

Consider a solution containing N_i ions of charge e_i in a total volume V, where $i = 1, 2, \ldots, s$. Locate two elements of volume dV_1 and dV_2 by vectors $\mathbf{r}_1$ and $\mathbf{r}_2$, drawn from an arbitrary origin, as shown in Figure 1. If we know that a j-ion is in dV_1, the concentration of i-ions in dV_2 will not equal $n_i = N_i/V$, the gross average, but will be $n_{ji}(\mathbf{r}_1, \mathbf{r}_{21})$, due to electrostatic and hydrodynamic interaction of the several ions. Let $\mathbf{v}_{ji}$ represent the (mean) velocity of an i-ion in dV_2 when a j-ion is in dV_1. Then, in terms of the symmetrical density functions

$$f_{ji}(\mathbf{r}_1, \mathbf{r}_{21}) = n_j n_{ji}(\mathbf{r}_1, \mathbf{r}_{21}) = n_i n_{ij}(\mathbf{r}_2, \mathbf{r}_{12}) = f_{ij}(\mathbf{r}_2, \mathbf{r}_{12}), \tag{1}$$

the general equation of continuity

$$\mathrm{div}_1 (f_{ij}\boldsymbol{v}_{ij}) + \mathrm{div}_2 (f_{ji}\boldsymbol{v}_{ji}) = 0 \tag{2}$$

must be satisfied. In the conductance problem, (1) becomes simply

$$f_{ji}(\boldsymbol{r}_{21}) = n_j n_{ji}(\boldsymbol{r}_{21}) = n_i n_{ij}(\boldsymbol{r}_{12}) = f_{ij}(\boldsymbol{r}_{12}), \tag{3}$$

because the distribution depends only on relative and not on absolute location. We retain the idealized picture of the solvent as a viscous dielectric continuum in which the motion of the ions obeys Brownian motion kinetics; inertial effects are assumed

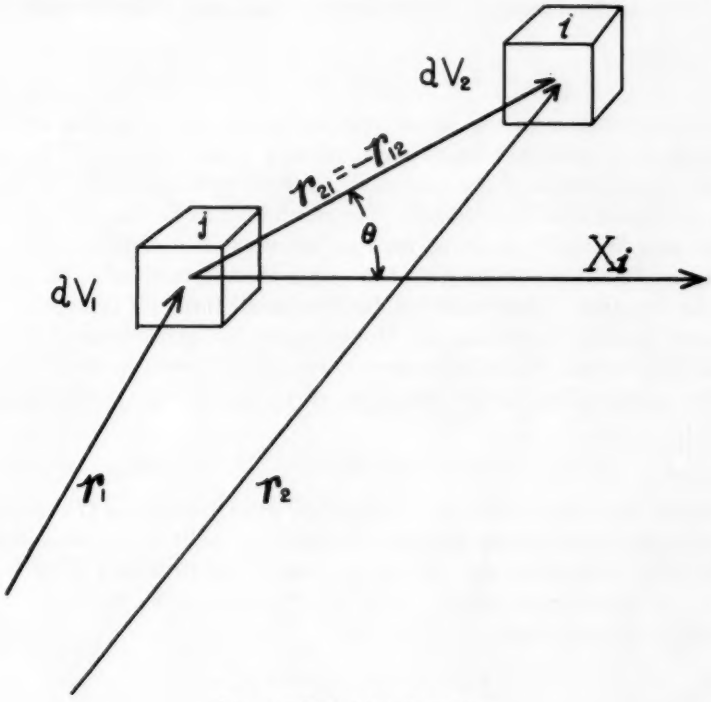

FIG. 1.—Definition of vectors

negligible. Accordingly, the pertinent properties of an ion of species j are its coefficient of friction $1/\omega_j$ and its electric charge e_j. In an electrical field X acting in the direction of the positive x-axis, the velocity of an isolated ion is therefore

$$\boldsymbol{v}^\circ_j = Xe_j\omega_j\boldsymbol{i}. \tag{4}$$

At finite concentrations, electrostatic interaction causes each ion to surround itself by an "atmosphere" carrying a compensating average charge density. Debye and Hückel[4] computed the distribution of this shielding charge for the static case and obtained the familiar limiting result

$$\psi^\circ_j(r) = \frac{e_j e^{-\kappa r}}{Dr} \tag{5}$$

for the average potential of the electrostatic field surrounding a point ion. Motion of the ions in an external electric field distorts the charge distribution, so that the above potential must be replaced by

$$\psi(r) = \psi^\circ(r) + \psi'(r).$$

The density functions (3) likewise consist of radial and unsymmetrical parts.

In previous work, it was assumed that atmospheric charge densities of neighboring ions could be superimposed; careful investigations have shown that this is a good approximation in the computation of ψ°. We have satisfied ourselves that this use of the superposition principle introduces only quite small errors (of the order of κ^2) in our final results. Accordingly, we assume for the force on an i-ion in dV_2

$$\boldsymbol{K}_{ji} = Xe_i\boldsymbol{i} - e_i\nabla_2\psi'_i(a) - e_i\nabla_2\psi_j(r_{21}). \tag{6}$$

This force contributes to the mean velocity of an ion according to (4). The Brownian motion produces an average velocity equal to $-\omega_i kT\ \nabla_2 \ln f_{ji}(r_{21})$. Finally, the local motion of the solvent is modified by the presence of another ion (with its atmosphere) in the vicinity. The resultant flow field $\boldsymbol{v}_i(r_{21})$ was computed by Debye and Hückel;[5] we have used an alternative derivation which permits the boundary conditions to be stated in terms of the potential in all but a small part of the function. Hence, except for this small term (of order c in the final conductance equation) involving the Stokes radius, the hydrodynamic terms in the relaxation field contain the same parameter a as the electrostatic ones.[6] Combining the various contributions to the velocity $\boldsymbol{v}_{ji}$ of an i-ion in the vicinity of a j-ion, we obtain

$$\boldsymbol{v}_{ji}(r_{21}) = \boldsymbol{v}_i(r_{21}) + \omega_i[\boldsymbol{K}_{ji} - kT\ \nabla_2 \ln f_{ji}(r_{21})], \tag{7}$$

with a similar expression for $\boldsymbol{v}_{ij}(r_{12})$. We then substitute (6) in (7) and the result in (2). The divergence terms (div $\varphi\boldsymbol{A} = \boldsymbol{A}\cdot$grad $\varphi + \varphi$ div $\boldsymbol{A}$) are expanded, noting that $\nabla\cdot\boldsymbol{v}_i = 0$. Functions are reduced to those of one variable $r = r_{21} = -r_{12}$ according to the operational rules $\nabla_2 = -\nabla_1 = \nabla$; $\nabla_1\cdot\nabla_1 = \nabla_2\cdot\nabla_2 = \Delta$. Potentials are assumed to be of the form

$$\psi_j(r_{21}) = \psi^\circ{}_j(r) + \psi'{}_j(r), \tag{8'}$$

$$\psi_i(r_{12}) = \psi^\circ{}_i(r) - \psi'{}_i(r), \tag{8''}$$

and the distribution functions are decomposed similarly:

$$f_{ji}(r_{21}) = f^\circ{}_{ji}(r) + f'_{ji}(r) = f_{ij}(r_{12}) = f^\circ{}_{ij}(r) - f'_{ij}(r). \tag{9}$$

The field strength is assumed to be below that at which any Wien effect is observable, so that $\psi' \sim X$, $f' \sim X$. In the above expansion, all terms in X^2 are therefore dropped; furthermore, the functions of spherical symmetry cancel each other because $\boldsymbol{v}_{ji} = \boldsymbol{v}_{ij} = 0$ when $X = 0$. The result of the above manipulation is

$$\begin{aligned}(e_i\omega_i - e_j\omega_j)X\mathbf{i}\cdot\nabla f^\circ{}_{ji}(r) - kT(\omega_i + \omega_j)\Delta f'_{ji}(r) - f^\circ{}_{ji}(e_i\omega_i\Delta\psi'_j - e_j\omega_j\Delta\psi'_i) - \\ (e_i\omega_i\nabla\psi'_j - e_j\omega_j\nabla\psi'_i)\cdot\nabla f^\circ{}_{ji} - f'_{ji}(e_i\omega_i\Delta\psi^\circ{}_j + e_j\omega_j\Delta\psi^\circ{}_i) - \\ (e_i\omega_i\nabla\psi^\circ{}_j + e_j\omega_j\nabla\psi^\circ{}_i)\cdot\nabla f'_{ji} + [e_i\omega_i\nabla\psi'_i(a) - e_j\omega_j\nabla\psi'_j(a)]\cdot\nabla f^\circ{}_{ji} - \\ (\boldsymbol{v}_i - \boldsymbol{v}_j)\cdot\nabla f^\circ{}_{ji} = 0.\end{aligned} \tag{10}$$

One boundary condition (impenetrability) arises from the requirement that the radial component of the relative velocity of any two ions in contact must vanish. Thus the sum

$$f_{ij}(\boldsymbol{v}_{ij}\cdot\boldsymbol{r}_{12}) + f_{ji}(\boldsymbol{v}_{ji}\cdot\boldsymbol{r}_{21})$$

has to vanish at $r = a$. With the convention $\boldsymbol{r}_{21} = \boldsymbol{r} = -\boldsymbol{r}_{12}$, this condition becomes

$$[(f_{ij}\boldsymbol{v}_{ij} - f_{ji}\boldsymbol{v}_{ji})\cdot\boldsymbol{r}]_{r=a} = 0. \tag{11}$$

Let $\boldsymbol{Y}$ represent the flow vector in the above bracket; then, by a calculation parallel to that which led to (10), we obtain

$$\begin{aligned}\boldsymbol{Y} = {} & (e_i\omega_i - e_j\omega_j)f^\circ_{ji}X\boldsymbol{i} - kT(\omega_i + \omega_j)\nabla f'_{ji} - f^\circ_{ji}(e_i\omega_i\nabla\psi'_j - e_j\omega_j\nabla\psi'_i) - \\ & f'_{ji}(e_i\omega_i\nabla\psi^\circ_j + e_j\omega_j\nabla\psi^\circ_i) + f^\circ_{ji}[e_i\omega_i\nabla\psi'_i(a) - e_j\omega_j\nabla\psi'_j(a)] - \\ & (\boldsymbol{v}_i - \boldsymbol{v}_j)f^\circ_{ji},\end{aligned} \tag{12}$$

where $(\boldsymbol{Y}\cdot\boldsymbol{r}) = 0$ at $r = a$.

It will be more convenient to use the boundary condition in the form

$$Z(a) = 0, \tag{13}$$

where

$$Z(r) = \frac{(\boldsymbol{Y}\cdot\boldsymbol{r})}{x}. \tag{14}$$

The following operations are used in expanding (14): $\boldsymbol{i}\cdot\boldsymbol{r} = x$, $x/r = \cos\theta$; $\boldsymbol{i}\cdot\nabla f^\circ(r) = \partial f^\circ/\partial x$; $\nabla f^\circ(r)\cdot\nabla\psi'(r) = (df^\circ/dr)\,(\partial\psi'/\partial r) = (df^\circ/dr)\,[\partial(\psi'/\cos\theta)/\partial x]$; $\boldsymbol{r}\cdot\nabla f'(r) = r\,\partial f'/\partial r = x\,d(f'/\cos\theta)/dr$; $\boldsymbol{r}\cdot\nabla\psi^\circ(r) = r\,d\psi^\circ/dr$; $(\boldsymbol{v}\cdot\boldsymbol{r})/x = v_r/\cos\theta$; $\boldsymbol{v}\cdot\nabla f^\circ(r) = v_r\,df^\circ/dr$. The result, after specializing to $s = 2$, is

$$\begin{aligned}Z(r) = {} & (e_1\omega_1 - e_2\omega_2)Xf^\circ_{21} - kT(\omega_1 + \omega_2)\frac{d}{dr}\left(\frac{f'_{21}}{\cos\theta}\right) - f^\circ_{21}\left\{e_1\omega_1\frac{d}{dr}\left(\frac{\psi'_2}{\cos\theta}\right) - \right. \\ & \left. e_2\omega_2\frac{d}{dr}\left(\frac{\psi'_1}{\cos\theta}\right)\right\} - \frac{f'_{21}}{\cos\theta}\left(e_1\omega_1\frac{d\psi^\circ_2}{dr} + e_2\omega_2\frac{d\psi^\circ_1}{dr}\right) + f^\circ_{21}\left\{e_1\omega_1\frac{d}{dr}\left(\frac{\psi'_1}{\cos\theta}\right)_a - \right. \\ & \left. e_2\omega_2\frac{d}{dr}\left(\frac{\psi'_2}{\cos\theta}\right)\right\} - (v_{1r} - v_{2r})\frac{f^\circ_{21}}{\cos\theta}.\end{aligned} \tag{15}$$

Similar substitutions are made in (10), and from the Poisson equations

$$\Delta\psi'_j = -\left(\frac{4\pi}{D}\right)\sum_i \frac{f'_{ji}e_i}{n_j} \tag{16}$$

we obtain, on specializing to $s = 2$,

$$\Delta\psi'_1 = -\frac{4\pi f'_{12}e_2}{Dn_1} = \frac{4\pi f'_{21}e_2}{Dn_1} \tag{16'}$$

and

$$\Delta\psi'_2 = -\frac{4\pi f'_{21}e_1}{Dn_2}. \tag{16''}$$

These equations serve to eliminate the potentials from a number of terms. In one of the inhomogeneous terms, the Debye-Hückel approximation

$$\Delta\psi^\circ{}_j = \kappa^2\psi^\circ{}_j \tag{17}$$

is used; here

$$\kappa^2 = \left(\frac{4\pi}{DkT}\right)(n_1e_1{}^2 + n_2e_2{}^2). \tag{18}$$

The final result is the equation of continuity translated into a form ready for integration:

$$(e_1\omega_1 - e_2\omega_2)X\left(\frac{\partial f^\circ{}_{21}}{\partial x}\right) - kT(\omega_1 + \omega_2)\Delta f'_{21} + \left(\frac{4\pi f^\circ{}_{21}f'_{21}}{D}\right)\left(\frac{e_1{}^2\omega_1}{n_2} + \frac{e_2{}^2\omega_2}{n_1}\right) -$$
$$\left\{e_1\omega_1\frac{\partial}{\partial x}\left(\frac{\psi'_2}{\cos\theta}\right) - e_2\omega_2\frac{\partial}{\partial x}\left(\frac{\psi'_1}{\cos\theta}\right)\right\}\frac{df^\circ{}_{21}}{dr} - \kappa^2 f'_{21}\,(e_1\omega_1\psi^\circ{}_2 + e_2\omega_2\psi^\circ{}_1) -$$
$$\left(e_1\omega_1\frac{d\psi^\circ{}_2}{dr} + e_2\omega_2\frac{d\psi^\circ{}_1}{dr}\right)\frac{\partial}{\partial x}\left(\frac{f'_{21}}{\cos\theta}\right) + \left\{e_1\omega_1\frac{\partial}{\partial x}\left(\frac{\psi'_1}{\cos\theta}\right)_a -\right.$$
$$\left. e_2\omega_2\frac{\partial}{\partial x}\left(\frac{\psi'_2}{\cos\theta}\right)_a\right\}\frac{df^\circ{}_{21}}{dr} - (v_{1r} - v_{2r})\left(\frac{df^\circ{}_{21}}{dr}\right) = 0. \tag{19}$$

Investigation[6] of (19) shows that the last five terms are of higher order in κ ($\sim c^{1/2}$) than the first three. We therefore define $F_{21}(\mathbf{r}) = h_{21}(r)\cos\theta$ as the solution of

$$(e_1\omega_1 - e_2\omega_2)X\left(\frac{\partial f^\circ{}_{21}}{\partial x}\right) - kT(\omega_1 + \omega_2)\Delta F_{21} +$$
$$\left(\frac{4\pi f^\circ{}_{21}F_{21}}{D}\right)\left(\frac{e_1{}^2\omega_1}{n_2} + \frac{e_2{}^2\omega_2}{n_1}\right) = 0, \tag{20}$$

subject to the boundary condition that the corresponding terms of (15) vanish at $r = a$:

$$\left[(e_1\omega_1 - e_2\omega_2)Xf^\circ{}_{21} - kT(\omega_1 + \omega_2)\left(\frac{dh_{21}}{dr}\right) -\right.$$
$$\left. f^\circ{}_{21}\left\{e_1\omega_1\left(\frac{d\Phi_2}{dr}\right) - e_2\omega_2\left(\frac{d\Phi_1}{dr}\right)\right\}\right]_{r=a} = 0. \tag{21}$$

Here $\Phi_j(r)\cos\theta = \Psi_j(\mathbf{r})$, where $\Delta\Psi_j = -(4\pi/Dn_j)\sum_i F_{ji}e_i$; in other words, Ψ_j and F_{ji} are our previous ψ'_j and f'_{ji}, but subject to boundary conditions for non-zero radius instead of for the vanishing radius which was previously used.

Since the potentials are determined by fourth-order differential equations, we need three more boundary conditions: they are $\Psi(\infty) = 0$, $(\partial\Psi/\partial r)_{r=\infty} = 0$ and $\Psi(a - 0) = \Psi(a + 0)$. The latter leads to

$$\left(r\frac{\partial\Psi}{\partial r} - \Psi\right)_{r=a} = 0. \tag{22}$$

Falkenhagen and coworkers[7] solved (20) using the same boundary conditions for

potential as those listed above, and using the Eigen-Wicke[8] distribution, but they chose a rather arbitrary fourth condition. Their result differs from ours by terms of order κ^2. In expanding (20) and (21), we use the Debye-Hückel approximation[4] for the unperturbed potentials around ions of finite size

$$\psi^\circ_j = \left(\frac{e_j e^{-\kappa r}}{Dr}\right)\left[\frac{e^{\kappa a}}{(1+\kappa a)}\right] \tag{23}$$

and the corresponding value for the distribution function

$$f^\circ_{21} \approx n_1 n_2 \left(1 - \frac{e_1 e_2 e^{-\kappa r}}{\mu DkT}\right), \tag{24}$$

where

$$\mu = (1+\kappa a)e^{-\kappa a}. \tag{25}$$

The following abbreviations

$$\gamma^2 = q^2\kappa^2 = \left(\frac{4\pi}{DkT}\right)\frac{n_1 e_1^2\omega_1 + n_2 e_2^2\omega_2}{\omega_1+\omega_2} \tag{26}$$

and the Bjerrum parameter

$$b = -\frac{e_1 e_2}{aDkT} > 0 \tag{27}$$

also aid in a compact formulation of the result.

The relaxation field ΔX is given by

$$-\Delta X = \mathrm{grad}_x\, \Psi(a). \tag{28}$$

Solution of (20), followed by solution of the corresponding Poisson equation and adjustment to the boundary conditions, gives finally,[6] on substitution in (28),

$$\frac{\Delta X}{X} = \frac{e_1 e_2 q^2\kappa}{3DkT(1+q)}\,\frac{1+(1+q)\,(1-[1+\kappa a]/b)\,(\kappa a/2)}{(1+\kappa a)\,(1+q\kappa a+q^2\kappa^2 a^2/3)}. \tag{29}$$

For convenience in later formulations, we write (29) in the form

$$\frac{\Delta X}{X} = \frac{e_1 e_2 q^2\kappa(1-\Delta_1)}{3DkT(1+q)} = -\alpha(1-\Delta_1). \tag{30}$$

It is immediately obvious that (29) differs from our earlier result by terms of order κ^2, which means that (29) will give c-terms in the conductance function. Now the five terms of (19) which we neglected above also give terms of order κ^2; still more important, they lead, in addition, to transcendental terms whose limiting behavior is like that of $(\kappa a \ln \kappa a)$. It is clear, then, that a conductance function, self-consistent to terms of order c, must also include the contributions of these orders from the neglected five terms. Furthermore, the latter terms in turn give rise to still higher terms, but consistency requires that only terms $O(\kappa a)$ and $O(\kappa a \ln \kappa a)$ be retained in the numerators of these quantities. Accordingly, we set

$$f'_{ji} = F_{ji} + g_{ji} \tag{31}$$

and

$$\psi'_j = \Psi_j + p_j \tag{32}$$

in the second and third terms of (19) and in the corresponding terms of the Poisson equation. In the leading (initial) inhomogeneous term, we carry one more term of the power series (24). Elsewhere, the approximation $f'_{ji} \approx F_{ji}$, $\psi'_j \approx \Psi_j$ is used. A similar procedure is followed in the velocity boundary condition. The result, after dropping higher terms and cancellation of terms of order κ which vanish by virtue of (20) and (21), is

$$\frac{n_2e_1e_2^2}{8\pi D(\mu kT)^2}\frac{\gamma^2 X}{\partial x}\frac{\partial}{\partial x}\left(\frac{e^{-2\kappa r}}{r^2}\right) - \Delta g_{21} + \gamma^2 g_{21}(r) - \frac{e_1e_2\gamma^2 e^{-\kappa r}F_{21}}{\mu DkTr} + \frac{n_2e_2\gamma^2}{4\pi\mu kT}\frac{d}{dr}\left(\frac{e^{-\kappa r}}{r}\right)\frac{\partial\Phi}{\partial x} - \frac{e_1e_2\kappa^2e^{-\kappa r}F_{21}}{\mu DkTr} - \frac{e_1e_2}{\mu DkT}\frac{d}{dr}\left(\frac{e^{-\kappa r}}{r}\right)\frac{\partial h_{21}}{\partial x} - \frac{n_2e_2\gamma^2}{4\pi\mu kT}\frac{d}{dr}\left(\frac{e^{-\kappa r}}{r}\right)\left(\frac{\partial\Phi}{\partial x}\right)_a + \frac{n_1n_2e_1e_2(v_{1r}-v_{2r})}{\mu Dk^2T^2(\omega_1+\omega_2)}\frac{d}{dr}\left(\frac{e^{-\kappa r}}{r}\right) = 0, \tag{33}$$

and

$$Z'(r) = \frac{-n_2e_2\gamma^2Xe^{-\kappa r}}{4\pi\mu kTr} - \frac{d}{dr}\left(\frac{g_{21}}{\cos\theta}\right) + \frac{n_2e_2\gamma^2e^{-\kappa r}}{4\pi\mu kTr}\frac{d\Phi}{dr} - \frac{e_1e_2h_{21}}{\mu DkT}\frac{d}{dr}\left(\frac{e^{-\kappa r}}{r}\right) + \frac{n_2\gamma^2D}{4\pi e_1}\left(\frac{d\Phi}{dr}\right)_a - \frac{v_{1r}-v_{2r}}{\omega_1+\omega_2}\frac{n_1n_2}{kT\cos\theta}. \tag{34}$$

No subscript is written on Φ in the above equations because $\Phi_1 = \Phi_2$. Solution of (33) subject to (34) vanishing at $r = a$ and to the appropriate boundary conditions for potential, followed by manipulation parallel to that which led to (29), gives a rather complicated expression[6] which simplifies considerably for symmetrical electrolytes (where $n_1 = n_2$, $e_1 = -e_2$, $q^2 = 1/2$).

The final result is

$$-\frac{\Delta X}{X} = \alpha(1 - \Delta_1 + \Delta_2) + \frac{\beta\Delta_3'}{\Lambda_0}$$

where α is the coefficient derived by Onsager[2] for the point charge model, Δ_1 is the change in $\Delta X/X$ produced by introducing finite ion size, Δ_2 is the change due to the electrostatic perturbation terms summarized in (33), β is the electrophoresis coefficient[2] and Δ_3' derives from the relaxation field due to the flow produced at the location of the reference ion by its neighbors. In order to be consistent with the order of terms retained in the original differential equation, terms of order κ^2a^2 and higher in the numerators of the various Δ's are dropped: the final values are:

$$\Delta_1 = \frac{(1+q)\kappa a}{2p_3(1+\kappa a)}\left(1 + \frac{1}{b}\right) \tag{35}$$

$$\Delta_2 = \frac{b(1+q)\kappa a}{p_2p_3(1+\kappa a)^2}\left\{\frac{11\sqrt{2}+3}{24} - \frac{T_0}{2}(1+\kappa a+2q\kappa a) + \frac{T_1}{8}(1+\kappa a+q\kappa a) + \frac{7T_2}{8}(1+q\kappa a)\right\} - \frac{(1+q)\kappa a}{2p_2(1+\kappa a)^2} \tag{36}$$

$$\Delta_3' = \frac{-b\kappa a}{p_2(1+\kappa a)^2}\left\{\frac{35-6\sqrt{2}}{192} - \frac{T_0}{8}(1+\kappa a+q\kappa a) + \frac{T_1}{32}(1+\kappa a) + \frac{7T_2}{32}\right\} + \frac{11\,\kappa a}{96p_2(1+\kappa a)^2} \quad (37)$$

where $p_1 = (1 + \kappa a + \kappa^2a^2/2)$, $p_2 = (1 + q\kappa a + \kappa^2a^2/4)$, $p_3 = (1 + q\kappa a + \kappa^2a^2/6)$, $T_0 = \mathrm{Tr}(\kappa a)$, $T_1 = \mathrm{Tr}[(1 + q)\kappa a]$ and $T_2 = \mathrm{Tr}[(2 + q)\kappa a]$. We are compiling tables of numerical values of the above functions.[6]

The transcendental function which appears in (37) is

$$\mathrm{Tr}\,(x) = e^x \int_x^\infty \frac{e^{-t}\,dt}{t}. \quad (38)$$

In the limit of very small values of x, $\mathrm{Tr}\,(x) \approx -0.5772 - \ln x$, but this expansion is useless in the range of concentrations where $\alpha\Delta_2\Lambda_0\sqrt{c}$ is experimentally observable.[9]

For symmetrical electrolytes, the $\mathrm{Ei}(2\kappa a)$ term in our previous[3] equation (4.2.10) vanishes in the electrophoretic correction to conductance, and the original[1] electrophoresis term β need only be divided by $(1 + \kappa a)$ to give a result consistent in order of κ with our new relaxation term (35). The final conductance equation can therefore be summarized by the following formula:

$$\Lambda = \Lambda_0 - \alpha(1 - \Delta_1 + \Delta_2)\Lambda_0\sqrt{c} - \beta(1 + \Delta_3)\sqrt{c} \quad (39)$$

where $\Delta_3 = \Delta_3' - \kappa a/(1 + \kappa a)$; all the terms which involve hydrodynamic effects are thus grouped together.

A comparison of (39) with experimental data is shown in Figure 2, where Shedlovsky's values[10] for the conductances of the alkali halides are shown as the open circles. By plotting values of Λ, calculated by (39) and Shedlovsky's values of limiting conductances, at $c = 0.05$ for a series of selected values of a, a value for a can be interpolated which reproduces the experimental value of Λ at this concentration. Then values of conductance at other concentrations were computed, using the interpolated value of a; these are plotted as solid circles in Figure 2. The agreement appears to be well within the experimental error of about 0.03 Λ-units. The a-values found are as follows: LiCl, 4.31×10^{-8}; NaCl, 4.20×10^{-8} and KCl, 4.23×10^{-8}. These seem quite reasonable, considering that all errors and approximations are absorbed in this single arbitrary constant. Our equation can be fitted to data at higher concentrations, but we do not believe that it has physical reality for $\kappa a > 0.3$, because not only do still higher terms from (2) begin to become significant, but also other physical complications which we have completely ignored begin to play a part. For example, as Eigen and Wicke[8] first pointed out, the fact that an ion and a solvent molecule cannot simultaneously occupy the same place becomes important.

Our present derivation shows that the upward curvature of the conductance curves of strong electrolytes can be accounted for theoretically merely by a refinement of the earlier calculation; the only change required in the physical model is the replacement of point charges by charged spheres. This, in turn, means that more realistic values of association constants can now be calculated from conduct-

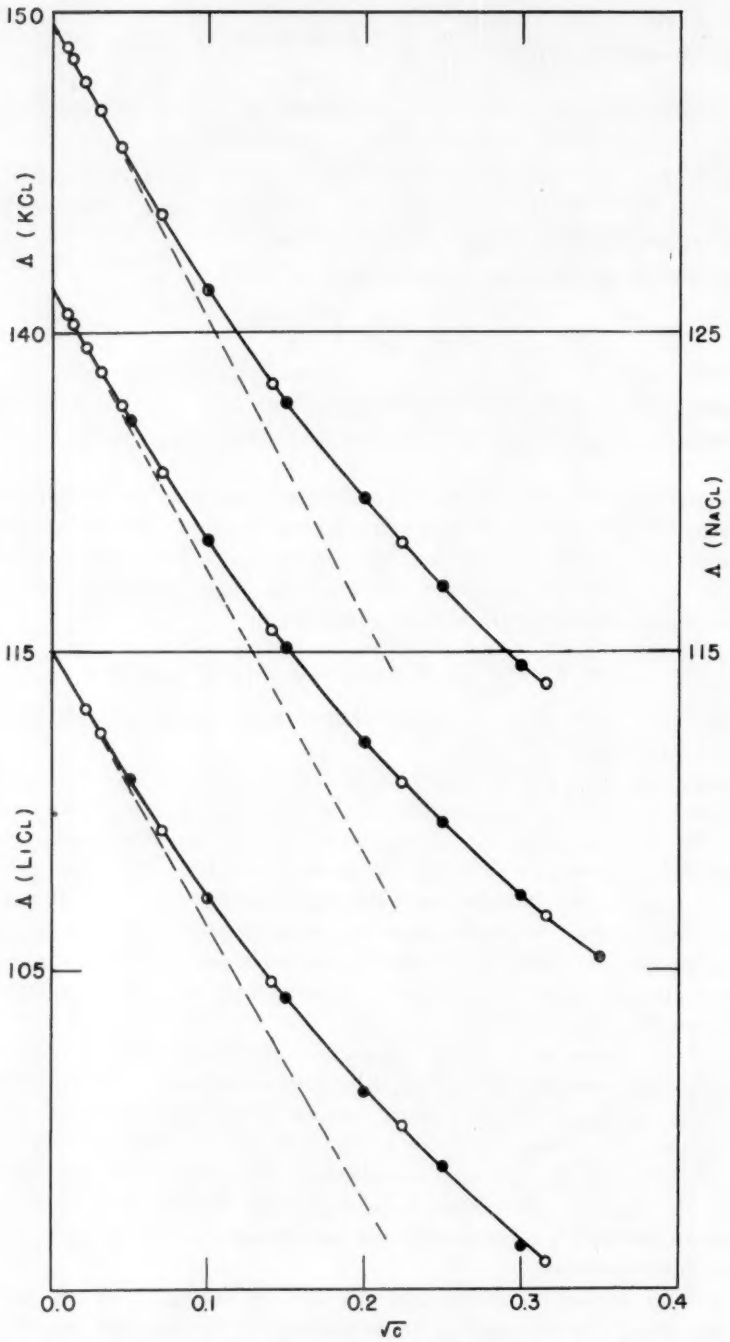

FIG. 2.—Comparison of experimental (*open circles*) and calculated (*solid circles*) values of conductance of the alkali halides

ance data than was previously possible in those cases where the fraction of ions associated is small. Work on this problem and on the case of unsymmetrical electrolytes is in progress.

* Contribution No. 1275.

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AN INTERACTION BETWEEN ALLELES AT THE Rh LOCUS IN MAN WHICH WEAKENS THE REACTIVITY OF THE Rh_0 FACTOR (D^u)

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Occasionally bloods are found which, when tested with different anti-Rh_0 (anti-D) sera, give some but not all the serological reactions expected from the presence of the Rh_0 (D) factor, or the intensity of such reactions is consistently weaker than the average. Owing to the clinical importance of the Rh_0 factor, on which the condition of Rh-positivity or Rh-negativity depends, such "intermediate variants" (Wiener[1]), usually symbolized as D^u (Stratton[2]) or $\mathfrak{R}h_0$ (Wiener[3]), represent a difficult problem for blood-grouping laboratories. For a review of the subject we refer to Race and Sanger.[4]

From a genetical point of view, a number of family investigations, actually not very large (Stratton and Renton;[5] Dunsford;[6, 7] Race, Sanger, and Lawler[8]), have led to the conclusion that the D^u variants are to be regarded as products of mutation of the *D* allele and are thus inherited like the other Rh blood factors. Owing to the fact that often the D^u variants in members of the same family show identical serological peculiarities, while they may differ broadly between different families, a series of D^u alleles has been supposed. This view received additional indirect support from the knowledge that generally blood groups behave as a direct, not mediate, product of the determining gene and are little influenced by other environmental or genetical agents.

While the interpretation given by the British authors is certainly true in some and may be true in the majority of D^u cases, other genetical interpretations cannot be disregarded.

In 1952 one of us,[9] discussing the data on some of the individuals now presented in family A (Fig. 1 and Table 2), stated that "the maternal chromosome *CDe* seems to produce in the offspring a normal D antigen when paired with the paternal chromosome *cde*, but a D^u antigen when paired with the paternal chromosome *Cde*." Thus the D^u phenotypes in that family appeared to be not the direct product of a mutant D^u (or $\mathfrak{R}^1$) gene but the result of interaction of the *D* chromosome with the partner *Cde* chromosome.

This observation was in agreement with the excess of CCD^ue phenotypes noted in their material by Race *et al.*,[8] who, following their hypothesis of an uncomplicated inheritance of D^u, were forced to attribute to such phenotypes the rare CD^ue/Cde genotype (the other possible alternative CD^ue/CD^ue should be exceedingly rare) because, on a serological level and in the absence of the hypothetical antiserum, D^u must be regarded to be dominant to *d* but recessive to *D* in the same way as A_2 is dominant to *O* but recessive to A_1. The authors concluded "that the relatively large number of CD^ue/Cde bloods demands an explanation but we have not any really satisfactory one to offer."[8]

Eldon[10] also observed an excess of CCD^ue individuals and suggested the existence of a modifying gene *U*. Although Eldon's views are not easily discussed on the basis of the scanty genetical and serological data which he presents, the hypothesis of a modifier linked with the Rh locus or independent of it cannot be discarded on the basis of present evidence.

An unusual opportunity for investigating this problem was presented when we found in a Roman Jewish community[11] an unusually high frequency of the *Cde* chromosome (*r'*gene), over 4 per cent as compared with less than 1 per cent in most Italian samples. Owing to the fact that we were dealing mainly with complete families, the genotypes were often ascertained unambiguously; out of 642 individuals tested, the *Cde* chromosome was present at least in the 43 following combinations: 24 *Cde/cde;* 16 *Cde/CDe;* 2 *Cde/cDE;* 1 *Cde/cDe.*

Moreover, according to the first Rh typing, where three different anti-D sera were routinely used in parallel, besides 545 Rh-positive and 83 Rh-negative persons, unequivocally classified as such, in fourteen cases a weakened or discordant D-positivity toward the three sera was observed, which was taken as sufficient evidence for classifying these bloods as D^u. In all cases these reactions, confirmed by quantitative titrations with several anti-D sera, were found in families in which the *Cde* chromosome was present, and thus Ceppellini's suggestion, previously quoted, was proved to be of more general value. Eleven such families (eight from the Roman Jewish community and three from other Italian districts) have now been studied. Six of these families were chosen because a propositus was identified as D^u in the first typing; in the other five cases the families were chosen through a Ccde propositus.

The presence of any weakly reacting form of the D factor was excluded for all bloods finally classified as Rh-negative (cde or Ccde) by means of the indirect antiglobulin test after sensitization with at least three (sometimes as many as nine) potent incomplete anti-D sera.

The level at which a D^u classification is justified is rather an arbitrary one; it is thus of fundamental importance in presenting such an investigation to state exactly the serological criteria and techniques used. For lack of space, such details will

be given only in the full account of our study, to be published elsewhere. In this preliminary note we shall present only a few examples of some of the families studied.

In Table 1 an example of titration is reported in detail for one family against one anti-D serum: according to the intensity of the agglutination, different point

TABLE 1

TITRATION AGAINST ONE ANTI-D SERUM (v) OF THE Rh-POSITIVE MEMBERS OF FAMILY A*

FAMILY MEMBERS	DILUTION OF ANTI-D SERUM (v) 1	2	4	8	16	32	64	128	SCORE
I-2	+	++^{v}	++^{e}	++^{e}	++^{e}	++	±	w	43
II-6	−	−	++	+	−	−	−	−	8
II-8	++	++^{v}	++^{e}	++^{v}	++^{v}	+	w	−	39
I-4	−	w	+	w	−	−	−	−	5
II-10	++	++^{e}	++^{e}	++^{e}	++^{e}	+	±	−	44

AGGLUTINATION SYMBOLS AND SCORING

++^{e}	= Complete agglutination into a few solid clumps	= 9 points
++^{v}	= Clumps easily recognized with the naked eye	= 7 points
++	= Large clumps seen under low-power mag.; rare unagglutinated cells	= 5 points
+	= Clumps of 8–15 elements, some unagglutinated cells	= 3 points
±	= Rare clumps of 4–6 elements, many unagglutinated cells	= 2 points
w	= Occasional clumps of 2–4 elements, majority of cells unagglutinated	= 1 point

* See Figure 1 and Table 2.
Note the prozone, most marked with the two weakly reacting bloods (II-6 and I-4).

scores are attributed for each dilution (from 10 to 1), and the sum of points makes up the individual score for a given serum. Usually at least five anti-D saline agglutinating sera for each set of experiments were used; the sera were selected only inasmuch as they did not contain any antibody other than anti-D; strict precautions for avoiding bias in reading were taken.

In Table 2 the scores for the members of family A (Fig. 1) are presented: the total scores (sum of the scores for the six different antisera) show that the reac-

TABLE 2

FAMILY A (FIG. 1): INDIVIDUAL SCORES OF THE Rh-POSITIVE MEMBERS AGAINST 6 DIFFERENT ANTI-D SALINE SERA (ALL Rh-NEGATIVE INDIVIDUALS RECHECKED WITH INDIRECT ANTIGLOBULIN TEST)

	PHENOTYPE*	GENOTYPE†	ANTI-D SERA (i)	(ii)	(iii)	(iv)	(v)	(vi)	TOTAL
I-1	Ccde	*Cde/cde*	...[*]	...	..	..	..	..	0
I-2	CcDe	***CDe/cde***	115	108	75	62	43	37	440
II-5	Ccde	*Cde/**cde***	...	...	..	..	..	..	0
II-6‡	CCD^{u}e	*Cde/**CDe***	64	62	23	44	8	18	219
II-7	cde	*cde/**cde***	...	...	..	..	..	..	0
II-8	CcDe	*cde/**CDe***	108	110	80	74	39	39	450
I-3	cde	***cde/cde***	...	...	..	..	..	..	0
I-4	CCD^{u}e	*Cde/CDe*	60	71	19	48	5	12	215
II-9	Ccde	*Cde/**cde***	...	...	..	..	..	..	0
II-10	CcDe	*CDe/**cde***	121	110	69	59	44	41	444

* Phenotypes symbolized by the letters corresponding to the blood factors actually found by using the 5 Rh anti-sera: anti-C, anti-c, anti-D, anti-E, anti-e; d symbolizes negativity toward anti-D. D^{u} reported when on the first typing an abnormally weak reaction of D was observed.
† Genotypes unambiguously determined from the family segregations; in the offspring the chromosome inherited from the father is printed in italic; the chromosome from the mother, in italic boldface.
‡ Propositus.

tivity of the D antigens produced by the same *CDe* chromosomes (2, 6, 8 and 4, 10) is markedly changed according to whether they are paired with a *cde* or a *Cde* chromosome.

It is worth while to emphasize that we aimed mainly at investigating whether the presence of a *Cde* chromosome in the genotype quantitatively depressed the reactivity of the D antigen, a fact which may not be related to the appearance of a true D^u phenotype as originally defined by Race *et al.*[8] Actually, while a few of our *D/Cde* individuals (see Tables 1 and 2) show a markedly different reactivity toward different anti-D sera (a typical feature of D^u phenotypes, which seems to be of a qualitative order), the majority of them merely score lower than normal D bloods with all anti-D sera. This suggests that the phenomenon here described is in the main a quantitative one.

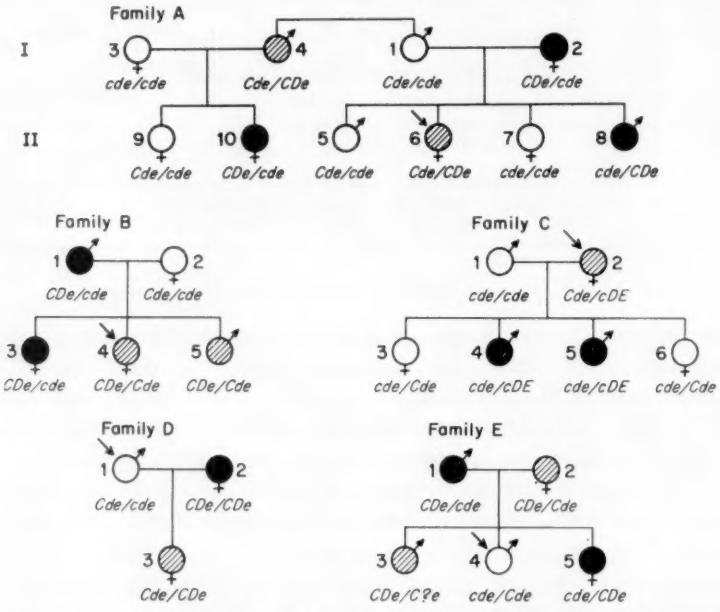

FIG. 1.—● = D-positive blood, of normal strength when titrated against several anti-D sera. ◐ = D-positive blood, significantly weaker than average on titration (like a D^u variant). ○ = D-negative blood (I.C.T.-negative, with several strong anti-D). → = Propositus. For family A see Table 2. For families B, C, D, and E see Table 3. In genotypes of offspring paternal chromosome is written first (*from Ceppellini et al.*[15]).

A proper answer to such a quantitative problem can only be obtained by subjecting the data to an analysis of variance, as presented in Table 3. The Rh-positive members of four families were titrated on two different days (experiments I and II) against the same five anti-D sera. The twelve individuals are divided into two classes, according to whether, in their genotype, unambiguously defined by family segregations, a *Cde* chromosome is present or not. Variability of reactions between individuals of the same genotype with respect to *Cde*–not *Cde*, tested on the same day, with the same serum, was used as the error: a t with 8 degrees of freedom is thus calculated for each serum (Table 3).

The statistical analysis proves beyond any doubt that in this experiment the five *D/Cde* genotypes show a lower D reactivity than the seven *D*/non-*Cde* geno-

types; the five different antisera show no significantly different discriminating abilities ($F_{4,40} = 1.93$).

Four similar sets of experiments have been analyzed, with the same results, that is, altogether, a total of 19 certain *D/Cde*, 20 *D*/not-*Cde*, and 4 *D/?* genotypes (detailed data to be published elsewhere).

We conclude that the presence of *Cde* paired to a normal *D* chromosome (in our investigation 15 *Cde/CDe*, 3 *Cde/cDE*, and 1 *Cde/cDe* genotypes were studied) produces a significant weakening of the D reactivity, which in extreme cases may be of the same order as the serological behavior shown by typical D^u bloods. Therefore, D^u phenotypes are not always due to the same genetical mechanism.

Such interpretation applies also to certain of the families reported by Race *et al.*[8] (families Lowe, Green, Curtis; probably also the two individuals Gillham and Pope—the last one a typical example of low-grade D^u) and by Eldon.[10]

TABLE 3

STATISTICAL ANALYSIS OF DATA

		Individual scores against 5 different anti-D sera					Total score
		I	II	III	IV	V	
Class D/Cde:							
1st day	D-3	62	43	18	43	56	222
	E-2	50	41	30	26	45	201
2d day	B-4	39	26	28	40	56	189
	B-5	37	34	32	36	66	206
	C-2	39	25	29	39	56	188
	$\bar{m}$	47.20	33.81	27.40	36.80	55.8	201.00
Class D/not-Cde:							
1st day	D-2	86	71	56	49	65	325
	E-1	68	56	42	51	67	284
	E-5	80	64	52	52	67	315
2d day	B-1	51	42	50	51	71	265
	B-3	66	45	45	55	62	273
	C-4	57	42	53	50	72	274
	C-5	54	41	52	52	72	271
	$\bar{m}$	66.00	51.57	49.71	51.43	68.00	286.71
	t (8 d.f.)	5.23*	6.54*	7.58*	5.39*	4.16†	11.21*

Statistical analysis of the scores representing the strength of agglutination of 12 Rh-positive individuals belonging to 4 families against 5 different anti-D saline sera (families B, C, D, E [Fig. 1]). The same panel of anti-D sera was used in the two experiments carried on on two different days. The individuals have been grouped into two classes according to the presence or absence of *Cde* in their genotype (*ascertained unambiguously through family segregations*). D-2 comes from two CcDe parents and has a cde sib. E-3, who scores 52, 35, 32, 34, 44 (total 197), has not been included in the analysis because of the uncertain genotype.

* Significant at .001 P level.

† Significant at .01 P level.

We do not discuss here the obvious practical implications of our findings, for instance, with regard to the use of Rh blood groups in paternity cases. We wish only to point out that, for the blood groups as for other characters, the phenotype cannot be regarded in any case as a direct reflection of the genotype.

From a formal point of view the phenomenon described could be explained in a variety of ways. We mention only the interpretations for which at least a basis for discussion exists:

a) If the weakening effect of *Cde* were confined to the CCD^ue phenotype, it could be regarded as due to a dosage effect between competing Rh alleles: two *C* alleles versus only one *D* produce D^u. The fact that the same phenomenon has been observed in *Cde/cDE* and *Cde/cDe* as well as in *CDe/Cde* genotypes does not justify such a hypothesis.

b) The weakening effect could be produced not by the *Cde* chromosome as a whole but by a mutant form of *d* (say, d^w) capable of interacting with a partner allele D. Up to now, this would have been recognized only in the Cd^we combination. It should be investigated whether a cd^we chromosome exists, that is to say, whether some CcD^ue or cD^ue phenotypes are to be regarded as CDe/cd^we or cDe/cd^we. Surprisingly enough, a number of published D^u families do not contradict, at least from a formal point of view, this possibility.

In conclusion, the weakly reacting D phenotype which occurs when a *Cde* chromosome is paired to a normal *D* chromosome is, in our opinion, one instance of the many interactions between alleles taking place at the Rh locus. Similar interactions between *CDe* and *cDE* (for example) have been assumed by Cameron *et al.*[12] to produce a depression of the anti-E reactivity. This kind of interaction appears more reasonable if we regard Rh as a *complex* locus, inherited as a unit but built up from more than one site of mutation (Ceppellini;[13] Dunn[14]).

Summary.—In eleven Italian families, eight from the "ghetto community" of Rome, a weakened reaction of the D antigen has been shown to be associated with the simultaneous presence of the chromosome *Cde* (*r'*) in the genotype. This is assumed to be due to interaction between *D* and the combined effects of *Cde* in juxtaposition or to other peculiarities of particular *Cde* chromosomes.

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*AN EXTREME CASE OF HETEROSIS IN A CENTRAL AMERICAN POPULATION OF DROSOPHILA TROPICALIS**

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Natural populations of many species of *Drosophila* flies are chromosomally polymorphic. Two or more gene arrangements in the same chromosome, differing in inversions of blocks of genes, occur in the chromosome pool of many populations. Since the carriers of the different gene arrangements interbreed freely, some of the wild flies are structural homozygotes (which have the two chromosomes of a pair with the same gene arrangement), while other flies are structural heterozygotes (the two chromosomes with different gene arrangements). In at least some natural populations the structural heterozygotes exhibit heterosis, and the chromosomal polymorphism is, therefore, balanced.[1] The mean adaptive values of the heterotic heterozygotes are superior to those of the homozygotes in the environments which the population inhabits. In many instances the homozygotes are less viable than the heterozygotes. If a differential mortality favoring the heterozygotes takes place between the egg and the adult stage, the adult fly population contains relatively more heterozygotes and fewer homozygotes than are demanded by the Hardy-Weinberg equilibrium equation.[1] However, it is naïve to think that heterosis must always lead to differential mortality in preadult life. Adaptive superiority of the heterozygotes may as well be due to a greater fecundity, to greater longevity, or to more intense sexual activity of the heterozygotes compared with the homozygotes. Furthermore, the heterosis may be more pronounced in one sex than in the other, at one season than at another, or in some habitats which the population occupies than in others[2]. Balanced polymorphism is maintained by natural selection so long as the heterozygotes retain an over-all adaptive advantage over the homozygotes.

The purpose of the present article is to describe an instance of balanced polymorphism in a natural population in which the fitness of the homozygotes is extremely low, in fact approaching zero. Nevertheless, the population not only does not appear to be on the verge of extinction but is remarkably prosperous. One may infer that the heterozygotes in this population are quite well adapted to their environments.

Natural Populations Sampled.—Mr. William B. Heed, of the University of Texas, very generously sent us living *Drosophila* flies of the *willistoni* species group which he collected in Honduras in April and in El Salvador in May and August, 1954, while he was working at the Instituto Tropical de Investigaciones Cientificas at San Salvador. Among these flies there were individuals of the four sibling species of the *willistoni* group, namely, *D. willistoni*, *D. equinoxialis*, *D. tropicalis*, and *D. paulistorum*.[3] The females collected in nature were placed singly in regular laboratory culture bottles with banana-agar food and allowed to produce offspring. Most of them were inseminated and produced good crops of larvae. When the latter were fully grown, the salivary glands of one larva from the progeny of each female were stained in acetic orcein, and the chromosomes were examined for

inversion heterozygosis. In the present article we are concerned only with the findings in *D. tropicalis*.

Four different configurations were observed in the left limb of the second chromosome (II L). Some larvae had no heterozygous inversions; others had a single subbasal inversion (represented schematically in the upper part of Fig. 1); others had a single submedian inversion (middle part of Fig. 1); still others had both inversions (lower part of Fig. 1). The subbasal inversion in our material is identical with that found by Townsend[4] in population samples from Haiti, the Dominican Republic, and Puerto Rico and pictured in his Figure 3. The submedian inversion lies apparently somewhat distally from the inversion shown in Townsend's Figures 1, 2, and 3. The subbasal and submedian inversions in our material are clearly independent, and an uninverted section can be seen between them in double heterozygotes (Fig. 1).

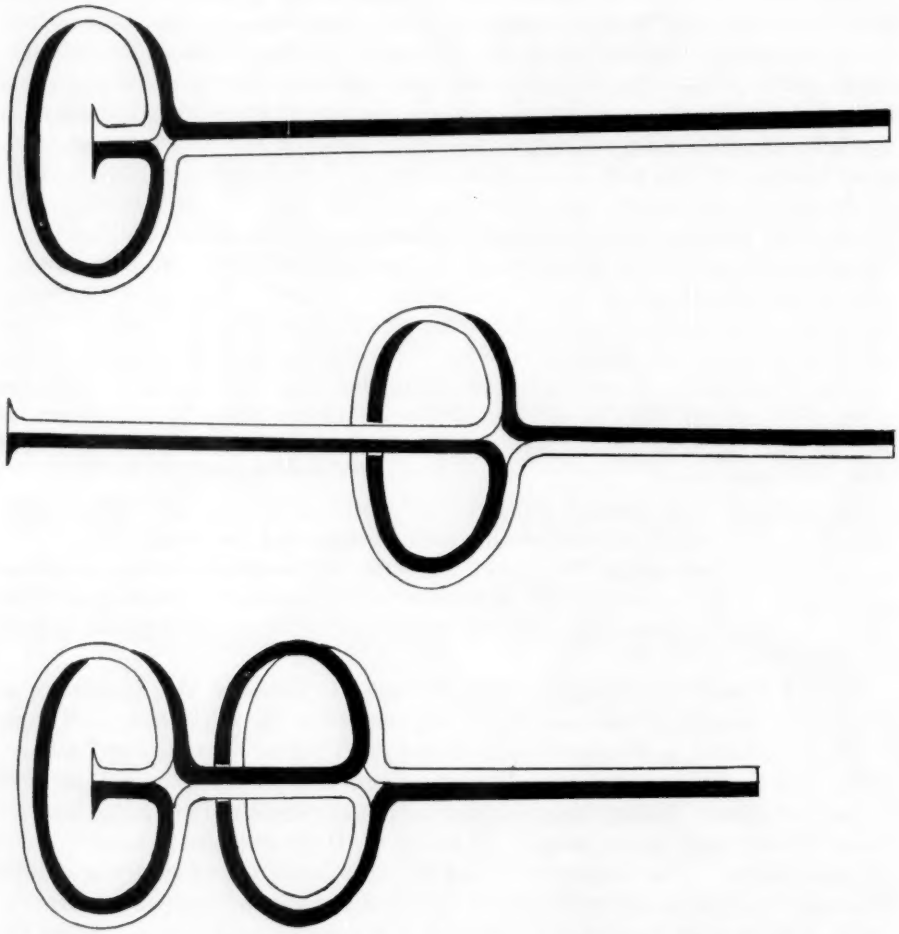

FIG. 1.—Schematic representation of the configurations in the second chromosome in heterozygotes for the subbasal inversion (*above*) for the submedian inversion (*middle*) and for both inversions (*below*).

Now, 74 of the females whose progenies we examined were collected by Mr. Heed in the vicinity of Lancetilla, Honduras; 6 near La Lima, Honduras; and 23 from the neighborhood of San Salvador. The chromosome configurations found by us were as shown in the accompanying tabulation. Among the 74 larvae from the

Locality	Free from Inversions	Subbasal Inversion	Submedian Inversion	Subbasal and Submedian
Lancetilla	18	45	4	7
La Lima	1	3	2	0
El Salvador	9	2	6	6

Lancetilla population, 52 or 70.3 per cent, were heterozygous for the subbasal inversion. This is significantly more than the 50.0 ± 5.8 per cent, which is the highest possible incidence of heterozygotes for a single inversion in a random-breeding Mendelian population in which the inversion homo- and heterozygotes are equally viable. It is most unlikely that the Lancetilla population of *D. tropicalis* has a breeding system which makes the two homozygotes mate preferentially to each other to produce heterozygous progenies. A more probable working hypothesis is that one or both inversion homozygotes are less viable than the heterozygotes and, consequently, that more heterozygotes than homozygotes survive to the late larval stage at which the chromosomes are studied.

This working hypothesis may apply only to the Lancetilla population. The El Salvador sample had only 8 subbasal inversions among 23 larvae examined, and the small La Lima sample had 3 heterozygotes among 6 larvae. According to Townsend,[4] the subbasal inversion is rare or absent in Cuba; and Dobzhansky, Burla, and Da Cunha[5] have not found it in Brazilian populations. Townsend (personal communication) found the subbasal inversion in progenies of 4 out of 10 females from the Dominican Republic. The observed frequency of the submedian inversion is in no population significantly greater than 50 per cent.

Experimental Population.—To test the above working hypothesis, an experimental population of *D. tropicalis* was set up in late May, 1954, in a wooden population cage of a type used in our laboratory for experiments with *D. pseudoobscura* and other species. The foundation stock of the experimental population consisted of the progenies of the Lancetilla females which hatched in the cultures from which the larvae were taken for the cytological examination the results of which are described above. Not all the cultures contributed equal numbers of parents to the foundation stock, but it is certain that this stock contained flies from most of the 74 Lancetilla cultures.

The population was allowed to breed freely for about 4 months in an incubator at 25° C. Samples of the eggs deposited in the cups of the population cage were then taken, and the larvae coming from them were raised under optimal conditions (with a refeeding with yeast) in culture bottles with banana food. Among the 150 larvae examined cytologically, the following situation was found:

Free from Inversions	Subbasal Inversion	Submedian Inversion	Subbasal and Submedian
14	111	0	25

As many as 90.7 per cent (136 out of 150) of the larvae were heterozygous for the subbasal inversion. It is evident that a differential mortality of the homozygotes occurs even under conditions of larval growth approaching optimum. Of

course, there must exist two kinds of inversion homozygotes among these larvae, having in duplicate each of the two gene arrangements which give rise to the subbasal inversion configuration. *Drosophila tropicalis* is, unfortunately, not good enough as cytological material to enable us to distinguish between these homozygotes. However, if only one of the homozygotes were lethal and the other were fully viable, we would observe at most 66.7 ± 3.9 per cent of heterozygotes. The actually observed figure is significantly higher.

The question naturally arises whether any homozygotes at all survive to the adult stage, particularly under the conditions of crowding and competition for food obtaining in population cages and, presumably, also in some natural habitats. Instead of taking samples of the eggs deposited in the population cage, we have next withdrawn from the cage some of the cups with the nutrient medium in which masses of pupae about ready to hatch were found. When the adults emerged from these pupae, virgin females and males were taken and outcrossed, in individual cultures, to flies of the Brazilian race of the strain descended from a wild female collected at Palma in the state of Goiaz, and at Içana in the state of Amazonas, by Professor C. Pavan and the senior author. These strains are structurally homozygous. A complication arises because the Honduran flies are subspecifically distinct from the Brazilian flies. As shown by Townsend,[4] the West Indies are inhabited by the subspecies *D. tropicalis cubana*, while in Brazil there occurs *D. tropicalis tropicalis*. The subspecies do not cross as easily as do strains belonging to the same race; the hybrids show a short submedian inversion (not identical with the submedian inversion observed in the Honduran and Salvadorean populations), and the F_1 hybrid males are sterile. The population of Lancetilla belongs to the subspecies *cubana*, and more than half the single-pair matings between the Lancetilla and Palma flies fail to produce offspring. The Lancetilla flies cross freely and produce fertile hybrids with flies from Cuba.

When the Lancetilla × Palma hybrid larvae are obtained, they show in the II L chromosome the submedian interracial inversion described by Townsend[4] and also one of the four following configurations: (A) a subbasal inversion, like Lancetilla flies; (B) a submedian inversion, like Lancetilla flies; (C) both the subbasal and the submedian; and (D) neither the subbasal nor the submedian. At least two larvae were examined cytologically from each of the Lancetilla × Palma cultures. If these two larvae showed different configurations in the II L chromosome, it was concluded that the Lancetilla parent (which developed, of course, in the population cage) was a heterozygote for two different gene arrangements in this chromosome. If the two larvae proved identical, further larvae were examined from the same culture, and usually a second configuration was found (in one instance, in the ninth larva examined). In a few cases (less than ten) the cultures contained so few good larvae that fewer than five of them could be classified as to the gene arrangement; these cases are not included in the count. The successful determinations are as shown in the accompanying tabulation (the two letters showing the gene arrangements in the two II L chromosomes of a pair).

	A/D	A/B	C/D	A/C	A/A	D/D	Total
Females	101	23	20	1	2	3	150
Males	33	5	10	1	1	0	50
Total	134	28	30	2	3	3	200

Only 8 out of the 200 adult flies tested (the A/C, A/A, and D/D classes) were homozygous for the gene arrangement in the basal part of the II L chromosome, and 192, or 96.0 per cent, were heterozygous for the subbasal inversion. This is slightly but barely significantly higher than the frequency found among the larvae raised under optimal conditions (the difference being 5.3 ± 2.7 per cent). It should also be noted that the submedian inversions occur equally frequently in combination with the gene arrangements in the basal part of the chromosome which do and which do not give a subbasal inversion in the hybrids between the strains from Lancetilla and from Palma (the A/B and C/D classes are about equal, namely, 28 and 30 flies out of 200).

Mortality of Eggs and Larvae.—An attempt was made to determine the stage of the life-cycle at which the differential mortality of the homozygotes occurs. Groups of about 50 adult flies from the experimental population were made to oviposit on the nutrient medium on paper spoons. The spoons were inspected daily, and the larvae that hatched were transferred by means of a needle to vials with food and left to develop at 25° C. Care was taken to use only the spoons which had not too many eggs (37–64). Similarly, not too few and not too many larvae were placed in any one vial (where possible, 20 larvae per vial).

Among the 1,135 eggs counted (on 23 spoons), 711, or 62.64 per cent, gave larvae. The egg mortality on different spoons was not uniform (chi square, 112.73), but on no spoon did all the eggs hatch. Among the 686 larvae which were allowed to develop, 623, or 90.8 per cent, gave adults. The larval survival was uniform (chi square, 23.6 for 22 degrees of freedom). In all, slightly more than 50 per cent of the eggs gave adult flies. In the absence of control experiments, it cannot be decided how many homozygotes were surviving among the flies that emerged. Since the egg hatch is rarely complete in normal laboratory cultures of *Drosophila*, some homozygotes were doubtless reaching the adult stage. Although most of the mortality occurs apparently in the egg stage, some larval and a small amount of pupal mortality also occur.

Comparison of Lancetilla with Other Populations.—The Lancetilla population of *D. tropicalis*, or at least its subpopulation in our experimental cage, approaches the status of a balanced lethal system. Close to one-half of the zygotes which these populations produce in each generation die on account of homozygosis for an unfavorable gene complex in the subbasal portion of the II L chromosome. Because of this differential mortality, the populations consist mostly of the heterotic inversion heterozygotes.

This amazing situation is certainly not characteristic of the species as a whole. Indeed, the sample of the Salvadorean population (see above) contained more than 50 per cent of homozygotes for the gene arrangements in the subbasal part of the chromosome. Although it is most likely that in this population the fitness of the structural homozygotes is lower than that of the heterozygotes, at least some of the homozygotes survive. Unfortunately, the progeny of the Salvadorean flies was discarded before its value was realized, and no experimental population of Salvadorean origin could be made. Townsend's data[4] suggest that, at least in Cuba, the heterozygosis for the subbasal inversion is not obligatory. And, finally, the Brazilian population lacks the subbasal inversion entirely and contains few inver-

sions of any kind. Does it follow that the Brazilian population is most prosperous and that that of Lancetilla is on the verge of extinction?

The available evidence indicates that just the opposite is true. In equatorial Brazil *D. tropicalis* is a rare species, far outnumbered by other species of the *willistoni* group (*D. willistoni*, *D. paulistorum*, and *D. equinoxialis*). *Drosophila tropicalis* has been found to be common in only one locality in Brazil,[5, 6] and that locality represents an ecologically highly specialized habitat (patches of forest among the swamps of northeastern Marajo Island). In the West Indies *D. tropicalis* seems to be widespread and sometimes more common than its sibling, *D. willistoni*.[4] Mr. Heed very kindly put at our disposal his records of *Drosophila*-collecting in El Salvador and Honduras. In El Salvador he found flies of the *willistoni* group to be fairly common in a variety of habitats, chiefly on various fruits. At Lancetilla Mr. Heed found the *willistoni* group to be predominant in the rain forest (56 per cent of the total catch of *Drosophila*) and fairly common in man-modified habitats. The *willistoni*-like flies which he sent us were classified as to species usually by examination of the chromosomes in their progeny and by inspection of the male genitalia, but sometimes by just one of these two methods (the examinations of the genitalia were kindly made and recorded for us by Mr. B. Spassky). The relative frequencies (in per cent) of the four sibling species in the samples were as shown in the accompanying table.

Locality	*tropicalis*	*willistoni*	*equinoxialis*	*paulistorum*	Flies Examined
Lancetilla	54.8	11.1	34.1	0	135
La Lima	85.7	0	14.3	0	7
El Salvador	17.9	35.4	44.7	2.0	246

At Lancetilla, *D. tropicalis* appears to be the most successful species of *Drosophila*. In El Salvador it surrenders the dominance to other species. In Brazil the species is rare. This certainly does not suggest that the loss of close to one-half of the zygotes produced has jeopardized the species at Lancetilla. On the contrary, the heterosis which has developed in the inversion heterozygotes has apparently conferred a high adaptedness on the population as a whole and has permitted it to attain ecological dominance in the environments which it has mastered. Balanced polymorphism does raise the fitness of the Mendelian population in which it occurs.

Wright[7] found by mathematical analysis that the spread of translocations which produce disturbances of the disjunction of chromosomes at meiosis in heterozygotes will be opposed by natural selection. This demonstration led other writers to cast doubts on the evidence that translocations play a part in the chromosomal evolution of sexual populations, such as those of *Drosophila*. Wright's analysis was, however, explicitly based on the assumption that the translocation heterozygotes have no intrinsic advantages over the homozygotes. The Lancetilla population of *D. tropicalis* shows that this assumption, though valid in most cases, cannot be simply taken for granted, as Wright has clearly pointed out in his work. In this connection it is useful to keep in mind that the situation found in the Lancetilla population is not quite unique. Berrie and Sansome[8] found that 40 individuals of *D. funebris* from Manchester, England, were heterozygous for a triple inversion in one of the chromosomes, and Buzzati-Traverso[9] has stated that inversion homozygotes are infrequent in populations of *D. subobscura* from several European countries. The

permanent heterozygosis in many strains of *Oenothera* is a classical case in the plant kingdom (see Stebbins[10] for review).

Summary.—A population of *D. tropicalis* from Lancetilla, Honduras, consists chiefly of heterozygotes for a certain inversion in the second chromosome. Inversion homozygotes are formed in every generation, but they die off chiefly in the egg stage and partly as larvae and pupae. The population thus sacrifices about one-half of its zygotes in every generation. The fitness of the heterotic heterozygotes is, however, so high that the population is a flourishing one. In certain other populations of the same species the inversions are less common or even altogether absent. These populations appear to be less prosperous than that of Lancetilla.

This work has been made possible by the courtesy of Mr. W. B. Heed, who sent us living *Drosophila* collected by him in Central America. Thanks are also due Mr. B. Spassky for classifying the flies and for maintaining the experimental population during our absence from the laboratory and to Dr. P. M. Sheppard for many valuable discussions.

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*THE EFFECT OF CHRONIC GAMMA RADIATION ON THE PRODUCTION OF SOMATIC MUTATIONS IN CARNATIONS**

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Introduction.—Chronic gamma radiation from a Co^{60} source has been found to be effective in producing gametic mutations of endosperm characters in maize. Also, the mutation rate increased more sharply than the increase in dose rate, giving an exponential relationship when mutation rate was plotted against dose rate.[1]

It was desired to test this finding with another organism and also to study mutations in somatic rather than germinal tissue. Carnations were chosen for this study. It was thought that somatic mutations in flower color could be easily

scored. Also, carnations are vegetatively propagated, and it should be possible to propagate any mutations involving an entire branch. This plant, which makes a rather rapid growth, should provide quickly information as to what might be expected from some of the slower growing woody plants, i.e., apples, peaches, cherries, berries, etc., that are being grown in the Brookhaven gamma field.

Materials and Methods.—The mutation rate was ultimately studied in three different carnation clones: William Sim (also known as "Red Sim") and two of its somatic mutants, Harvest Moon and White Sim, the last-named variety being used in both years. William Sim has the genetic constitution *Yy Ii AA SS rr mm*, while the genotypes of the other two clones are unknown. Work has been done on the constitution of White Sim, and it has been found to be a periclinal chimera with the germ tissue having the same genetic makeup as William Sim.[2] The flowers are white, since the subepidermal tissue contains no anthocyanin. On the basis of the study of Mehlquist and Geissman[3] of the factors determining flower color in the carnation, the genotypes of the white and orange mutants are postulated as follows: William Sim (red), *Y I A S r m;* White Sim (white), *y I A S r m* (epidermal constitution); Harvest Moon (orange), *Y i A S r m*.

Plants of the three stocks were grown at various distances from a Co^{60} source, as described by Sparrow and Singleton.[4] The plants were in the radiation field for the major portion of their growth, including flower-bud formation. Phenotypic changes in kinds and amount of pigment present in either the upper or the lower epidermal cells of the petals were scored for a period of a month after the plants had received more than two months' radiation. For example, red spots appeared on white flowers and on orange flowers. These mutant color areas were arbitrarily divided into size classes, which included spots, two sizes of streaks, and sectors of petals and of flowers. Each of the mutations was scored as a single event without regard to the number of cells or petals involved. In the case of sectors, in which the mutation always affected both the upper and lower epidermal layers of the petal, sectors appearing approximately opposite each other on both sides of the petal were scored as one event. In White Sim each instance of the red sinus blotch was scored once whether the blotch was found on one petal or at the attachment point of two petals.

No inhibitors have been found which give rise to white color.[5] However, since it could be argued that White Sim is a special case (the genetics not being known), it was decided to grow other carnation clones in the radiation field in 1954 to see whether they were likewise capable of mutating from recessive to dominant in the absence of a chimeral condition. It was found that Puritan and Achilles, both *y*-whites (*y I A* as opposed to *y I a* or *Y I a*), produced flushing under the influence of radiation. In addition, Puritan also showed pink streaks. A third variety, Northland, of the genotype *y I a*, produced faint flushes very rarely in the radiation field and not at all in the absence of radiation.

An additional red variety, Tetra Red, which is a mutant of William Sim containing both diploid and tetraploid tissue, was grown in the radiation field in 1954, to make comparisons with the type of changes found in the William Sim variety. Limited observations were made on somatic mutations in the field; also, plants were observed after removal from the field, as was done for all varieties grown in both years.

Results.—The three varieties investigated most intensively were White Sim, William Sim (Red Sim), and Harvest Moon (Orange). White Sim produced red and

flush mutations. The types of changes which occurred on William Sim will be discussed separately. The third clone, Harvest Moon, mutated to red (*Y I A*) and to cream (*y I A ?*), although only the red mutations are shown in the data, it being too difficult to detect all cream-colored areas with certainty. Significantly, the red mutations were practically all within the smallest size class (spots). Rarely did streaks greater than 2 mm. in length occur. In contrast, the cream-colored areas were observed quite frequently in the larger size classes.

Table 1 gives a summary of somatic mutations observed during the growing season. The doses ranged from 1.2 to 210 r/day. The incidence of "sinus blotch" is also recorded for the White Sim variety but not included under "total mutations," since the sinus blotch showed a completely different response to the radiation. At 50 r/d the rate was doubled for the White Sim variety. The variety William Sim showed a doubling of the control rate at 23 r/d. The variety Harvest Moon showed a doubling of the rates for the low doses at 23 r/d, although the lowest dose of radiation was higher than the control rate. The difference is not significant statistically.

Figures 1, 2, and 3 show graphs of the mutation rate plotted against dose for the three varieties. The rate of mutation of red to white is seen to be much greater than the reverse rate for white to red.

Types of Changes Observed on William Sim.— William or Red Sim mutated, primarily to white, pink, and dark red. However, numerous other changes were recorded, as follows:

True purple: These sectors appear purple to the naked eye and consist of tissue, some of the cells of which actually contain a reddish-purple pigment in part of the vacuole. The pigment may or may not be crystallized out in the center of the cell. *False purple or deep cherry-red:* This type may appear purple to

TABLE 1

AVERAGE NUMBER OF SOMATIC MUTATIONS PER FLOWER IN 1954*

RADIATION (R/DAY)	WHITE SIM VARIETY					WILLIAM SIM VARIETY (RED)						HARVEST MOON (ORANGE)	
	Flowers Scored	Sinus Blotch	Flush	Red	Total	Flowers Scored	White	Variegated	Pink	Dark Red	Total	Flowers Scored	Red
210	13	4.4	1.0	6.8 ± 2.6	7.8	11	141 ± 28	1.4	22.5	6.4	171	2	46.5 ± 6.1
160	25	5.3	0.56	6.0 ± 1.5	6.6	21	101 ± 16	1.7	21.0	3.6	127	13	24.3 ± 4.1
103	29	9.5	0.24	3.2 ± 0.8	3.4	23	67 ± 7.3	1.0	14.6	1.8	84	7	14.9 ± 2.2
50	31	11.0	0.06	2.4 ± 0.7	2.5	15	50 ± 8.6	0.20	10.5	1.4	62	17	6.2 ± 2.2
23	20	9.1	0.00	1.6 ± 1.8	1.6	12	28 ± 6.9	0.50	8.9	0.67	37.9	10	3.5 ± 2.0
11.4	32	8.6	0.03	1.2 ± 0.5	1.2	15	23.7 ± 4.3	0.27	5.5	0.93	29.4	5	1.6 ± 2.5
4.8	39	8.1	0.05	1.1 ± 0.3	1.2	27	18.2 ± 1.9	0.19	4.8	1.2	24.4	8	1.6 ± 1.4
2.2	33	9.0	0.00	1.3 ± 0.6	1.3	23	18.6 ± 4.1	0.52	6.2	0.70	25.0	12	1.3 ± 0.7
1.2	35	8.7	0.00	0.9 ± 0.3	0.9	19	17.9 ± 2.3	0.21	4.7	0.63	22.4	11	1.2 ± 0.7
Control	65	9.8	0.00	1.2 ± 0.3	1.2	31	14.3 ± 2.1	0.06	4.0	0.77	19.1	7	0.4 ± 1.5

* Confidence intervals ($t(0.5) \times$ S.E.) at .05 level.

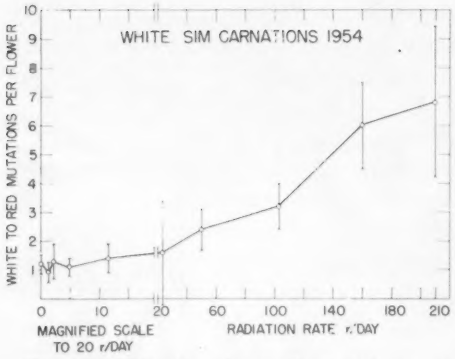
WHITE SIM CARNATIONS 1954
WHITE TO RED MUTATIONS PER FLOWER
0 10 20 60 100 140 180 210
MAGNIFIED SCALE TO 20 r/DAY
RADIATION RATE r/DAY

FIG. 1.—White to red somatic mutations per flower in White Sim variety.

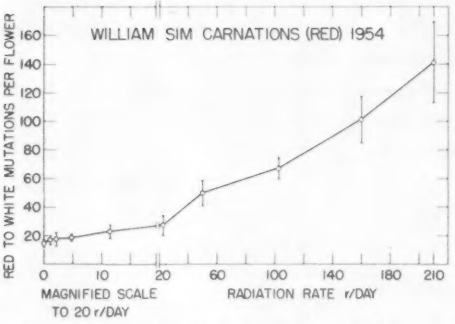
WILLIAM SIM CARNATIONS (RED) 1954
RED TO WHITE MUTATIONS PER FLOWER
0 10 20 60 100 140 180 210
MAGNIFIED SCALE TO 20 r/DAY
RADIATION RATE r/DAY

FIG. 2.—Red to white somatic mutations per flower in William Sim variety.

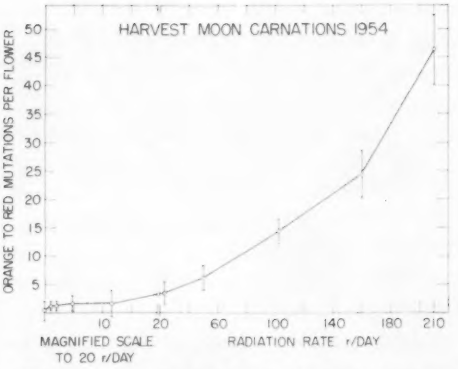
HARVEST MOON CARNATIONS 1954
ORANGE TO RED MUTATIONS PER FLOWER
10 20 60 100 140 180 210
MAGNIFIED SCALE TO 20 r/DAY
RADIATION RATE r/DAY

FIG. 3.—Orange to red somatic mutations per flower in Harvest Moon variety.

the naked eye, but under the microscope it is shown to consist of two types of cells in different proportions, deep cherry-red and bright red.

Dark red: Dark red and bright red cells make up this type.

Heavy variegated: This sector contains the same cells as found ordinarily in William Sim but in a more aggregate pattern.

Pink: The pink sector contains reddish-pink and pink cells.

Light variegated and light pink: These sectors are made up of white cells and cells of different intensities of pink.

White: There are two types of white sectors, both consisting almost entirely of white cells. The most common white sector has sharp outlines, like all the above sectors and like the red mutations on White Sim. The other type is very infrequent and has diffuse outlines, showing evidence of the movement of some substance into or out of the sector.

Orange: One mutation to orange occurred.

White-outlined: This sector consisted of pigmented tissue surrounded by a band of two to three cells of white color.

In Table 1 data are presented for the mutations to white, pink, dark red, and variegated (heavy and light included together). The purple sectors were found to show no increase with increase in radiation over the control rate and hence are not considered radiation-induced events. It is not known whether this applies to both the true purple and the deep cherry-red sectors. All that is known is that there is a class of phenomena which was included with the purple sectors and which is insensitive to radiation.

Postirradiation Data.—Extensive postirradiation observations were made only on the variety White Sim from plants grown in the radiation field in 1953. In two independent instances, entire branches with red flowers arose on white plants after removal from the radiation field at 340 r/day. Cuttings were taken from these "mutated" branches, and the plants which developed bore only red flowers.

In addition to these two cases, cuttings were taken from the plants growing at the six highest dosages after these plants had received three and one-half months of irradiation in the field. Eleven of the eighty-seven plants which bloomed during a period of four months of observation showed mutations to red. In one case the mutation was expressed as part of a flower; a second case consisted of one red flower. The remaining mutations had affected whole branches of flowers. The latter were easily propagated and produced plants with red flowers. From thirty-five plants growing at the rows closest to the source, eight independent mutations of the propagable type were recorded. The flowers on these red plants or branches showed in every case a number of white streaks similar to that ordinarily found on flowers of William Sim. In view of the chimeral nature of the white plants, however, it cannot be stated whether changes to red are the result of mutations or of histological abnormalities. There is little doubt that they are radiation-induced.

A more striking phenomenon, however, was that the radiation had deleted the red sinus blotch from sixty-six of eighty-five plants which continued to produce white flowers. Except for three cases in which the plants were probably sectorially chimeral for the absence of the blotch, these plants consistently produced the same type of flower, either blotched or blotchless, during the entire four months of observation. Cuttings taken from the blotchless plants produced blotchless flowers.

It is apparent from the above and also from one case in which a flower showed a clear-cut demarcation of blotched and blotchless tissue that the sinus blotch is a heritable characteristic. The following observation suggests a genetic change of the subepidermal tissue. The blotchless white flowers do not show the peculiar injury reaction of White Sim flowers, in which, upon destruction of epidermal cells, pigment is formed by the cells beneath. (These flowers do, however, exhibit another, more common wounding reaction in which the epidermal cells themselves form the pigment as a consequence of wounding.) It is suggested that these blotchless plants are no longer chimeral for white-over-red. It is further suggested that the blotch is a character produced by the outcropping of subepidermal tissue at the margins of the petals, so that in the absence of the chimeral condition the blotch is not manifested. These ideas have not yet been subjected to genetic tests. Many instances of flush were observed, including at least one whole branch mutation to a flushed pink condition.

Only limited observations were made on plants removed from the 1954 irradiation field. One plant of Tetra Red, which was removed from the 8-meter row, where it had been receiving 645 r/day, was grown in the greenhouse. In February, 1955, one branch of this plant produced variegated red and white flowers, similar to the "Mamie" variety, a mutant type derived from William Sim.

More data are needed to determine a dose-response relationship for whole branch mutations appearing after the material has been removed from the radiation field. However, the preliminary results indicate that gamma radiation has been effective

in producing mutations that can be propagated. Thus it seems feasible to produce somatic mutations in other vegetatively propagated crops, including woody plants, some of which are now being grown in the Brookhaven gamma field. The Co^{60} gamma source may well become an important tool for the plant breeder.

Summary.—Carnations grown under conditions of chronic gamma radiation produced somatic mutations which can be vegetatively propagated. These mutations are white to red flowers, red to variegated, and standard double- to single-flowered types. For somatic mutations produced on flowers during radiation, it is not possible to state whether there is a linear relationship between mutation rate and dose rate. In one variety, Harvest Moon, there is a strong indication of an exponential relationship, but the other two varieties show no significant deviation from a linear relationship.

* Research carried out at Brookhaven National Laboratory under the auspices of the United States Atomic Energy Commission.

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*SPECULATIONS ON GENE ACTION AND PROTEIN SPECIFICITY**

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By definition, genes must possess two fundamental properties. First, they must be capable of self-reproduction, and, second, they must be able to direct the organization and development of an organism. While it has long been known that the carriers of the genetic material, the chromosomes, consist of deoxyribonucleic acid (DNA) and protein, it is only recently that evidence has accumulated which strongly suggests that the genetic specificity resides partly and perhaps wholly in the nucleic acid portion.[1] Watson and Crick[2] have proposed a model for DNA which beautifully accounts for the exactness of the replication process. The present paper deals with the second property of the gene—its mode of action. The problem to be considered is how a nucleic acid molecule can confer its specificity to a protein. The discussion will be limited to nucleic acids in general, since the same problem holds for both DNA and ribonucleic acid (RNA).[3] In each case specificity probably resides only in the arrangement of four bases (pyrimidines-cytosine and thymine or uracil, and purines-guanine and adenine). Protein specificity, on the other hand, must reside in part in the arrangement of some twenty amino acids. A simple relation between the arrangement of bases in nucleic acid and of amino acids in protein cannot be established without imposing certain restrictions on the possible order of amino acids in proteins.

It is obvious that there cannot be a 1:1 relation between a specific base and a specific amino acid, since there are too few bases. The situation can be improved

somewhat by postulating that the amino acids fit into cavities formed by adjacent bases which protrude from the nucleic acid chain, like rungs of a ladder. The bases that form the cavity would specify which amino acid will be located at that position. The sixteen cavities defined by all possible pairs of the four bases will not accommodate all the amino acids; furthermore, use of two adjacent bases to designate an amino acid imposes certain restrictions on the possible arrangement of amino acids in a protein. Given one amino acid, there are at most only eight others which could lie adjacent to it, since one of the bases which designates the adjacent amino acid is already established. Also, there is only one possible amino acid which can occur between any two amino acids, since the two bases which designate the central amino acid are already set by those on either side. These restrictions do not hold, as can be determined from an examination of the protein structures listed in Table 1. It should be pointed out that in the Watson-Crick model the spacing of the bases along the fiber axis, 3.4 A, is practically the same as the spacing of amino acids in an extended polypeptide chain—3.5 A. For this reason we are not considering the possibility that two adjacent amino acids are fitted into cavities formed by two adjacent pairs of bases.

TABLE 1

SEQUENCES OF AMINO ACIDS IN PROTEINS

*Insulin, fraction A:**

Gly–Ileu–Val–Glu–Glu–CyS–CyS–Ala–Ser–Val–CyS–Ser–
Leu–Tyr–Glu–Leu–Glu–Asp–Tyr–CyS–Asp

Insulin, fraction B:†

Phe–Val–Asp–Glu–His–Leu–CyS–Gly–Ser–His–Leu–Val–
Glu–Ala–Leu–Tyr–Leu–Val–CyS–Gly–Glu–Arg–Gly–Phe–
Phe–Tyr–Thr–Pro–Lys–Ala

β-Corticotropin:‡

Ser–Tyr–Ser–Met–Glu–His–Phe–Arg–Try–Gly–Lys–Pro–
Val–Gly–Lys–Lys–Arg–Arg–Pro–Val–Lys–Val–Tyr–Pro–
Ala–(Gly–Glu–Asp)§–Asp–Glu–Leu–Ala–Glu–Ala–Phe–Pro–
Leu–Glu–Phe

* F. Sanger and E. O. P. Thompson, "The Amino Acid Sequence in the Glycyl Chain of Insulin," *Biochem. J.*, **52**, iii, 1952.

† F. Sanger and H. Tuppy, "The Amino Acid Sequence in the Phenylalanyl Chain of Insulin. 2. The Investigation of Peptides from Enzymic Hydrolysates," *Biochem. J.*, **49**, 481–490, 1951.

‡ P. H. Bell, "Purification and Structure of β-Corticotropin," *J. Am. Chem. Soc.*, **76**, 5565–5567, 1954.

§ The exact sequence of amino acids listed in parentheses is in doubt.

Gamow,[4] using the double-stranded structure of the Watson-Crick DNA model, has postulated that particular amino acids fit into diamond-shaped cavities formed by combinations of the four bases, arranged two on each strand, in such a way that that one of the diagonals of the diamond corresponds to the hydrogen bond between a purine on one strand and pyrimidine on the other. His scheme creates twenty different cavities but still imposes a number of improbable restrictions. For one, as Gamow has shown, twelve of the twenty amino acids can be adjacent to only seven different amino acids, and only eight can be adjacent to as many as fourteen, since contiguous diamonds have bases in common. Furthermore, although many of the diamonds (amino acids) can occur as pairs, there are only four which can occur as pairs once removed, i.e., with another amino acid inserted between. These are P × P, R × R, T × T, and V × V, according to Gamow's

designation. In the few proteins analyzed there are already five amino acids occurring in the condition described.

It thus appears that there is not sufficient information carried by a section of a nucleic acid molecule to specify all the amino acids in a protein of equal length. We should, therefore, investigate the possibility that the nucleic acid confers its specificity to a protein by designating the spacing or arrangement of only a certain few amino acids. Naïve considerations with regard to structural similarities between some of the amino acids and the bases, in addition to a definite sequential pattern of the amino acids with cyclic side chains in the insulin fraction B molecule, have led to the postulate that the sequence of bases in nucleic acid determines the positions occupied by the aromatic amino acids (including histidine) in the protein molecule.

If we start at the pair of phenylalanine residues in the insulin fraction B molecule and consider the number of aliphatic residues which are separated by the cyclic amino acids, we find the sequence 0, 0, 1, 2, proceeding to the C terminal end. If we resume at the N terminal end, the sequence continues as 3, 4, 5, 7. Thus the cyclic amino acids appear to be spaced according to a definite pattern in this molecule. It is also of some interest to fold the chain of fraction B so that the cyclic amino acids are always located on the edge of the folds, as is shown in Figure 1. In such a folded configuration the aliphatic amino acids which

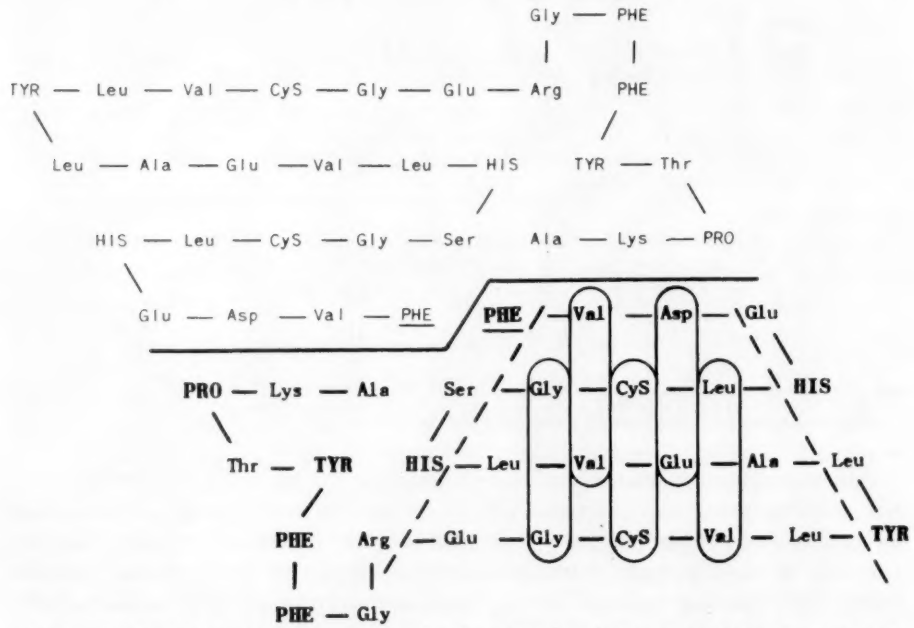
Gly — PHE
TYR — Leu — Val — CyS — Gly — Glu — Arg PHE
Leu — Ala — Glu — Val — Leu — HIS TYR — Thr
HIS — Leu — CyS — Gly — Ser Ala — Lys — PRO
Glu — Asp — Val — PHE
PHE — Val — Asp — Glu
PRO — Lys — Ala Ser — Gly — CyS — Leu — HIS
Thr — TYR HIS — Leu — Val — Glu — Ala — Leu
PHE Arg — Glu — Gly — CyS — Val — Leu — TYR
PHE — Gly

FIG. 1.—Folding and arrangement of two molecules of fraction B of insulin so that the amino acids with cyclic side chains (*in caps*) occur at the edge of the folds. The terminal phenylalanines are underlined. Notice the orderly arrangement of the aliphatic amino acids.

are found under the outer shell formed by the cyclics (inside the dotted line) apppear to be placed in quite orderly fashion. There are columns composed of 2 glycine, 2 valine, 2 cystine, another of glutamic and aspartic, which are quite similar, and yet another including leucine and valine, which are closely related amino acids. Over and above this, there appears to be

some periodicity in the sequence of these columns under the shell. If we consider glutamic and aspartic as being in the glutamic group, leucine and valine as being in the leucine group, and glycine and alanine as the glycine group, the group order reads: Glu–Leu–Gly–Leu–CyS–Glu–Leu–Gly–Leu. A similar pattern has not as yet been uncovered for the other protein molecules.

It is postulated that the planar rings of the aromatic amino acids would be differentially bound in cavities formed by two adjacent bases in the nucleic acid chain. The ability of purines to form complexes with aromatic compounds has long been known.[5] Specifically, phenylalanine and tyrosine, the aromatics with only six-membered rings, are thought to fit between two pyrimidines, the bases which have only six-membered rings. Tryptophan and histidine, which contain five-membered rings, would be bound by two purines, bases which have an iminazole ring (five-member) in addition to the pyrimidine ring. The distance between the adjacent phosphorus atoms along the sugar-phosphorus backbone is approximately 7 A, according to the DNA model of Watson and Crick. The bases lie perpendicular to the fiber axis. If the model were modified slightly so that the bases are tilted or inclined to the fiber axis (perpendicular to the sugar-phosphorus backbone), the distance between the bases would also be 7 A. Thus the interatomic spacing under these conditions would be of the correct order, 3.5 A, for resonance bonding between the aromatic amino acids and the bases which form the cavities (Fig. 2). The structure of RNA is not sufficiently known to permit similar considerations. The peptide chain is pictured as running down the center of the nucleic acid helix, with the R groups protruding toward the bases. The peptide linkage distance would thus correspond to the distance between the bases in the fiber axis, 3.4 A.

According to this hypothesis, phenylalanine or tyrosine will be bound wherever a cavity is formed by two adjacent pyrimidines on the nucleic acid chain. Histidine or tryptophan will be bound by two purines lying side by side. The sites of cavities composed of a purine and a pyrimidine correspond to the nonaromatics. There is more than enough information in the purine-purine combinations and the pyrimidine-pyrimidine combinations to specify the four aromatic amino acids, since there are four possible arrangements for each combination. Proline has a ring configuration but was not included with the aromatics because, being devoid of double bonds, it would not undergo resonance bonding with the bases. Furthermore, proline is an imino acid, and it is hard to imagine how it could fit into a cavity, since it would occupy a position in the polypeptide chain

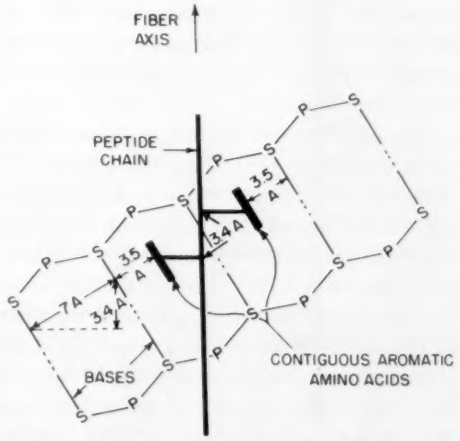

FIG. 2.—Diagrammatic representation of nucleic acid–protein complex (see text). The DNA should be envisioned as being spiralized around the peptide chain. *P* = phosphorus; *S* = sugar.

perpendicular to the bases. In the remainder of this paper, for simplicity, proline will be classed with the aliphatics.

The proposed hypothesis imposes certain restrictions on the arrangement of the amino acids. These restrictions involve the positions which the aromatics can occupy in the protein molecule with respect to each other. For example, since tyrosine is bound in a cavity formed by two pyrimidines and histidine in a cavity formed by two purines, these two amino acids cannot occur side by side. They must, in fact, be separated by an odd number of aliphatics. Similar considerations hold for either of the phenylalanine-tyrosine group with either of the tryptophan-histidine group. An even number of zero aliphatics must occur between aromatics when both are of the phenylalanine-tyrosine group and, similarly, when both are of the tryptophan-histidine group.

It is now possible to designate the bases (in terms of pyrimidines or purines) which occur in the nucleic acid template on either side of the aromatics in those proteins for which the amino acid sequence is known, and to check the spacings with those which are imposed by the hypothesis. As an example, the fraction A molecule of insulin will be used. This protein has two aromatics, both of which are tyrosine. Starting with the first tyrosine from the N terminal end, the sequence of bases must be $_{py}Tyr_{py}$. An aliphatic amino acid, glutamic acid, follows; hence the next base must be a purine, and the sequence must be $_{py}Tyr_{py}Glu_{pu}$. This must be so, according to the hypothesis, since, if this next base were also a pyrimidine, the cavity would be formed by two pyrimidines, and an aromatic of the tyrosine or phenylalanine class would be bound at that point. The next three amino acids are also aliphatics, which means that the base sequence on the nucleic acid chain must be $_{py}Tyr_{py}Glu_{pu}Leu_{py}Glu_{pu}Asp_{py}$. The next amino acid is an aromatic. Since one of the bases which will form the cavity is already established, according to this hypothesis, and is a pyrimidine, only an aromatic of the phenylalanine-tyrosine group can occupy that position, and, in fact, that aromatic is tyrosine. If the second tyrosine were one residue closer to or farther from the first, the scheme would not hold.

The same procedure can be followed for all the proteins containing more than one aromatic amino acid where the sequence has been established. In insulin fractions A and B and in β-corticotropin it is found that the aromatics tyrosine, histidine, and tryptophan always fall in the correct position as specified by the hypothesis. There are eight chances for them to be in either the right or the wrong place, and in each case they are right. Phenylalanine is also consistent; however, it is consistently wrong (except for the single instance where two phenylalanines are found together in the insulin B molecule). Phenylalanine appears to occur 180° out of phase, and this suggests a modification of the scheme. Up to this point we have considered only one of the strands in the nucleic acid helix. Let us now consider the doubled helix and assume that the protein is synthesized partly on one and partly on the other strand. Thus we visualize that protein synthesis is initiated along one of the strands and that phenylalanine represents the position where a switch is made onto the second strand (Fig. 3). At the next phenylalanine, the switch is made back to the first strand, with the end result that each of these sections begins with a phenylalanine. The switching could perhaps be associated with the breaks in the chain of DNA which Dekker and Schachman[6] have postulated. Protein synthesis

would proceed down one strand until it came to a break in the chain. At that point the switch would be made to the other chain. One would have to postulate that the second switch is not made when two phenylalanines occur together. If phenylalanine always occurs at a cavity opposite a break in the other chain, this aromatic amino acid would always appear 180° out of phase. Such a scheme would make it impossible for breaks to occur at the same level in both strands on the Watson-Crick model, as could happen if the breaks occurred at random.

The point is made that the genetic material confers specificity to a protein by designating the spacing and arrangement of the aromatic amino acids in the protein molecule. However, the arrangement of the aliphatics in a protein molecule is also quite specific and constant. How can this specificity be conferred? A number of hypotheses can be formulated. One possibility is that the specificity governing the order of the aliphatics is conferred partly by the arrangement of the aromatics on the nucleic acid template and partly by the cytoplasm (the specificity of the cytoplasm may, in turn, be under genic influence); the idea is that in a specific cytoplasm a limited number of specific di-, tri-, and polypeptides composed wholly of aliphatics are formed by some transpeptidation mechanism.[7] These peptides would then be fitted onto the template in the regions between the bound aromatic amino acids. The particular peptides which would be seated in any one region would be specified by the combination of aromatics and their spacing, and perhaps also by the sequence of bases on the nucleic acid template between the positions occupied by the aromatics. There are eight possible combinations for each spacing. The combinations of aromatics separated by an odd number of aliphatics are Tyr-His, Tyr-Try, Tyr-Phe, Phe-His, Phe-Try, Phe-Phe, His-Tyr, and Try-Tyr. Aromatic combinations separated by an even number of aliphatics are Tyr-Tyr, Phe-Tyr, His-His, His-Try, His-Phe, Try-Try, Try-His, and Try-Phe. In other words, the cytoplasm would specify the peptides which would be formed, whereas the nucleic acid–aromatic amino acid complex would specify which of these would be fitted onto the template. In the proteins analyzed, there are no instances in which a particular combination of aromatics separated by a set number of aliphatics occurs more than once. There are, however, three cases in

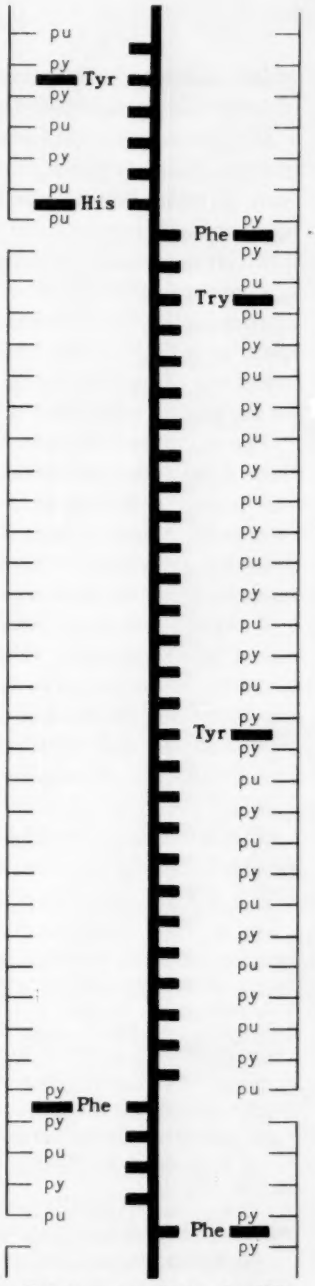

FIG. 3.—β-corticotropin. Diagrammatic representation of the nucleic acid–protein complex. The central heavy core represents the protein molecule. Short bars are aliphatics; long bars are labeled aromatic amino acids. Outer lines represent the nucleic acid doubled helix; *pu* = purine; *py* = pyrimidine.

which aromatics are separated by a trio of aliphatics, and these are similar in that in each case the third aliphatic is glutamic acid.

In some respects this scheme is a combination of the two general hypotheses of protein synthesis which have been championed, template versus transpeptidation,[8] each of which has its merits. It allows for direct genic control of protein specificity and also for the rapid rate of synthesis by having some of the amino acids laid down on the template already in the form of peptides.

Another possibility which might be considered in relation to the order of the aliphatics is that the nucleic acid chain is much longer than the protein chain which it specifies and hence that the order of the amino acids is determined by a large number of repeating units on the nucleic acid chain, these repeated units being delimited by the pattern of spacing of the aromatic amino acids.

The amino acid sequence of many more protein molecules must be known before these admittedly speculative hypotheses can be adequately tested. Such information is slow in appearing, for it involves much difficult and tedious labor, but it is essential for further insight into the problem of gene action. It is not to be expected that every protein will fit this scheme; some changes can and proably are made by transpeptidation.

Summary.—A hypothesis on the template mechanism of protein synthesis is discussed which proposes that the sequence of bases in nucleic acid determines the positions of the aromatic amino acids in the protein molecule. These amino acids are considered as being differentially bound in cavities formed by adjacent bases, phenylalanine and tyrosine being bound by two pyrimidines and tryptophan and histidine by two purines.

The interest and assistance of my colleagues in the Biology Division are gratefully acknowledged.

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*INDUCED POLLEN LETHALS FROM SEEDS OF DATURA STRAMONIUM EXPOSED TO DIFFERENT RADIATIONS**

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Types and patterns of pollen lethals in *Datura stramonium*, as induced by different radiations, were studied for the purpose of determining relative biological efficiencies and responses. Previous reports by the authors have given the effectiveness of thermal neutrons[1] and of fast neutrons from a nuclear detonation[2] in producing pollen aberrations. The results herein recorded are comparisons of mutation types, rates, and sectoring of plants arising from *Datura* seeds exposed to (1) X-radiation, (2) thermal neutrons, and fast neutrons from (3) a cyclotron and from (4) a nuclear device.

Analysis of mutation rates as indicated by induced pollen abortion in *Datura* has demonstrated a marked difference between the relative biological efficiency (RBE) of detonation neutrons and either gamma or X-radiation.[2] Fast neutrons from a nuclear device apparently induce the same qualitative types of pollen lethals as gamma rays or X-rays, but the sensitivity ratio, or RBE, is probably 13 or 14. A dose estimate of 760 rep of detonation neutrons compares with 10,000 r of either gamma of X-radiation. Detonation and cyclotron neutrons, considering the difficulties of dosimetry, have not been shown to possess significantly different biological efficiencies.[3] Thermal neutrons have a somewhat lower effectiveness; their RBE is about 11 for the production of certain chromosomal aberrations in *Tradescantia*.[4]

As in previous studies, radiation effects are evaluated in terms of pollen lethality.[1, 2] The rates and types of pollen abortion give, with considerable accuracy, the relative biological efficiencies and responses. Pollen abortion in *Datura* also affords a convenient means by which large numbers of plants can be analyzed for both qualitative differences and aberrant sectors. A companion article, concerned with the chromosomal condition of these plants, is in press.[5]

This present study includes data from a report on Operation Upshot/Knothole, Projects 23.4–23.14 and 23.16, of the United States Atomic Energy Commission, entitled *Genetic Effects of Fast Neutrons from Nuclear Detonations*, by Dr. H. H. Plough *et al.*, dated January, 1954.

Experimental Procedures and Results.—Seeds employed in these tests were of *D. stramonium*, Line 1. This line has been brought through a haploid generation and selfed for over forty generations. All seeds were dried to a moisture content of 4.9 per cent before exposure to radiation. Four separate lots of seeds were exposed to X-radiation, thermal neutrons, and fast neutrons from a cyclotron and from a

nuclear detonation. A fifth lot of seed constituted a portion of the untreated control. No seeds were used from flowers that yielded more than 1 per cent bad pollen. Seeds exposed to detonation neutrons were shielded from blast, heat, and much of the gamma radiation, as previously described by Spencer and Blakeslee.[2] These seeds, treated in 1953, were kept under optimum conditions of moisture and temperature until planted in the spring of 1954. Fast-neutron dosages from a cyclotron were made at Oak Ridge National Laboratory. The dose estimate of cyclotron neutrons was 650 rep, and the gamma contamination was estimated to be about 10 per cent of the total dose. Irradiation by thermal neutrons was carried out at Brookhaven National Laboratory, where the seeds were exposed to a flux of 7.09×10^8 neutrons for 185 minutes. The total dose or number of neutrons received by the seeds was approximately 7.82×10^{12}. Brookhaven National Laboratory also furnished the X-ray exposure of 10,000 r for one lot of seed.

The seeds were sown in flats and germinated in a greenhouse. Surviving plants were transferred to the field, where they matured and were studied for types of pollen abortion and for chromosomal aberrations.

Flowers were collected at random as the pollen matured. No specific number of flowers per plant was analyzed; however, the long duration of flowering made it possible to examine over twenty flowers from a substantial number of plants. These large collections, a total of almost 6,000 flowers, provide considerable data not only on pollen aberrations within flowers but also on mutant sectors. The pollen condition of flowers and plants is recorded against two-, four-, and sixteen-flower samples from the forks of the plant, as well as being based on the total random collection. These multiple collections were made because of the dichotomous development of the *Datura* plant and to uncover differences that might arise from sampling methods.

Three conditions or types of grains were recorded: (1) normal grains; (2) chromosomal types of pollen lethals, characterized by small, shriveled, and empty grains; and (3) gene types of lethals, seen as subnormal-to-very-small-size pollen, often showing degenerative changes in the character of its contents. Other pollen conditions (e.g., diploid grains) were recognized, but, inasmuch as they accounted for less than 1 per cent of the pollen in any flower, they are not reported.

Germination of the seeds and maturation of the plants were notably good and uniform for all treatments except for those seeds exposed to thermal neutrons (Table 1). Approximately 90 per cent of the seeds exposed to X-rays, to cyclotron neutrons, and to detonation neutrons germinated, and 4–6 per cent of the seedlings died before maturation. Unfortunately, the total dose of thermal neutrons (7.82×10^{12}) was much too severe to yield the comparable results for germination and survival intended. Comparisons with other work on thermal-neutron effects show this lethality to be a function of the dose.[2, 6] Spencer and Blakeslee have demonstrated that a total dose of 6.62×10^{12} neutrons (a flux of 4.6×10^8 for 4 hours) will allow over 75 per cent of the seeds to germinate but that 86 per cent of the subsequent plants will bear pollen lethals. Coincidently, this figure for pollen abortion and the percentage reported here for the thermal-neutron group are identical (Table 2).

Detonation neutrons of 760 rep induced 69 per cent of the plants to manifest abnormal pollen and 56 per cent of the flowers on these plants to show the same condition (Table 2). These data compare with the percentages yielded by X-radiation:

55 per cent of the plants and 53 per cent of the flowers contain bad pollen. The lower dosage of cyclotron neutrons induced fewer pollen lethals. Only 32 per cent of the plants and 27 per cent of the flowers contained aborted pollen. Too few plants (17) survived the heavy thermal-neutron treatment to allow meaningful use of these data.

Analysis of the results on types of pollen lethals induced in *Datura* by these different radiations is complicated by the fact that some treatments are not equivalent. However, the large size of the samples and the magnitude of the effects allow definite conclusions to be drawn from some of the trends. Decided quantitative differences are evident; yet there appears to be no such qualitative variation or differential response among samples exposed to either type of fast neutrons or to X-rays (Table 3). Ratios of chromosomal type to gene type of pollen abortion are quite alike for these three exposures. These ratios, *ca.* 4:1, seem to hold whether the data are based on a random sample or on a two- or four-flower sample from each

TABLE 1

GERMINATION OF *Datura* SEED

Exposure	Seed Exposed	Germination	Maturation
X-ray (10,000 r)	300	270 (90%)	258 (86%)
Thermal neutrons (7.82×10^{12})	300	149 (50%)	17 (5.7%)
Fast neutrons cyclotron (650 rep)	300	272 (90%)	254 (85%)
detonation (760 rep)	400	357 (89%)	333 (83%)
Control*	(300)	274 (91%)	265 (88%)

* Compiled from three control groups.

TABLE 2

INDUCED POLLEN LETHALS IN *Datura*

Exposure	Plants Tested	Plants with Pollen Lethals	Flowers Tested	Flowers with Pollen Lethals
X-ray (10,000 r)	245	135 (55%)	1,537	660 (53%)
Thermal neutrons (7.82×10^{12})	14	12 (86%)	28	25 (86%)
Fast neutrons cyclotron (650 rep)	247	78 (32%)	1,520	404 (27%)
detonation (760 rep)	326	223 (69%)	1,955	1,101 (56%)
Control	150	0 (0%)	579	0 (0%)

plant. If the flowers with both chromosomal and gene types of abortion are included, the ratios are decreased, but in approximately the same magnitude. Random collections from X-irradiated plants yielded 31 per cent, 7 per cent, and 5 per cent for chromosomal, gene, and the combination, respectively. A similar method of sampling gave percentages of 19, 5, and 3 for cyclotron neutrons and 39, 8, and 9 for detonation neutrons. No lethals were found in the 579 flowers examined of the control.

The small sample of flowers collected from the surviving thermal-neutron–treated plants indicate that a biologically different response may have been induced in this group. Percentages of 41, 17, and 28 for chromosomal, gene, and the combination (chromosomal and gene), respectively, were found for the slow-neutron dosage. The ratio of chromosomal to gene types of pollen lethals is therefore 3:1. A 3:2 ratio (20 flowers to 13) results if the combination is apportioned between the two single-type categories.

Estimates and comparisons of relative mutation rates may be furnished by data on pollen lethal sectors induced in *Datura*. These sectors generally develop from aberrant initials in the second layer of the embryonic plumule and occur as limited regions of the plant. At times the entire plant may be involved. Inasmuch as mature *Datura* plants may bear scores or even hundreds of flowers, the amount or degree of sectoring uncovered depends, to a large extent, on the size of the sample analyzed. Therefore, an attempt was made to obtain one flower from each half-plant and one and four flower collections from each quadrant (Table 4). When the method of sampling is increased from two flowers per plant to four flowers, the sectoring found is approximately doubled. An increase to sixteen-flower-per-plant samples shows a relatively small increase in sectoring. Apparently most sectors on plants with both good and bad pollen areas involve approximately one-fourth to one-half of the plant. Sectors smaller than one-fourth of a plant are comparatively rare. The similarities among percentages of sectoring of X-irradiated and fast

TABLE 3

TYPES OF INDUCED POLLEN LETHALS IN *Datura*

EXPOSURE	FLOWERS TESTED*	PER CENT FLOWERS WITHOUT POLLEN LETHALS	POLLEN LETHAL TYPES FOUND IN INDIVIDUAL FLOWERS (PER CENT)		
			Empty (Chromosomal)	Small (Gene)	Empty and Small (Chromosomal and Gene)
X-ray (10,000 r)	a— 1,537	57	31	7	5
	b— 492	63	27	8	4
	c— 920	64	30	6	4
Thermal neutrons (7.82×10^{12})	a— 29	14	41	17	28
	b—(insufficient data)				
	c—(insufficient data)				
Fast neutrons cyclotron (650 rep)	a— 1,520	73	19	5	3
	b— 496	81	14	3	2
	c— 960	80	15	3	2
detonation (760 rep)	a— 1,955	44	39	8	9
	b— 654	47	35	9	8
	c— 1,192	48	35	9	8
Control	a— 579	100	..	..	..

* a—Based on total flowers from random collection. b—Based on 2 flower-per-plant sample (one from each main fork). c—Based on 4 flower-per-plant sample (one from each quadrant).

neutron types show that different qualitative responses or biological differences are not induced in *Datura* by these dosages.

Discussion and Conclusions.—Analyses of pollen lethals evident in *Datura* plants developing from seeds exposed to radiations clearly demonstrate a marked difference for the relative biological efficiencies of fast neutrons from a nuclear detonation and fast neutrons from a cyclotron as compared with X-radiation. Detonation neutrons and cyclotron neutrons apparently induce the same sort of biological responses but are much more effective, thereby resulting in high ratios of aberrations per unit dose. The exposure of *Datura* seeds to cyclotron neutrons was not enough to give results equivalent to the X-ray dosage; however, the data on pollen lethal types show the qualitative effects provoked by detonation neutrons, cyclotron neutrons, and X-rays to be the same. Fast neutrons from a detonation have a relative biological efficiency of 13 or 14. This figure for the RBE of detonation neutrons is the same as that previously reported by Spencer and Blakeslee and is in agreement with the figure obtained by Kirby-Smith and Swanson and others.[3, 7]

A dose of fast neutrons from a nuclear device produced the same quantitative and qualitative effects as an equal dose from a cyclotron.[8] The same relative sensitivity ratio (*ca* 13:0) for the two kinds of fast neutrons is provided by the data both from chromosomal types of pollen lethals and from gene types. Apparently the same ratios are caused by the different exposures.

The relative effectiveness of thermal neutrons has been reported to be about 11 or 12 times the efficiency of X-radiation as measured by cytogenetic change.[4] The results reported here and previously[1] allow no definite conclusions to be drawn concerning relative effectiveness of thermal neutrons as compared to other radiations; however, a qualitative difference or response is shown.

Ratios of chromosomal types of pollen lethals to gene types are the same (*ca.* 4:1) for the X-irradiated and fast-neutron–treated materials but quite different (3:2) for the thermal-neutron dose. Biologically different responses for thermal neutrons as compared with X-rays have been noted by other investigators.[6, 9] Unlike these other observers, who noted gross morphological changes as different

TABLE 4

INDUCED POLLEN LETHAL SECTORS IN *Datura*

Exposure	Plants with Pollen Lethals	Plants with Same Pollen Lethals in All Flowers Collected*	Percentage of Sectoring between Chromosomal/Normal Type	Percentage of Sectoring between Gene/Normal Type	Percentage of Sectoring between Chromosomal/Gene Type
X-ray (10,000 r)	135	a—76 (56%)	28	11	5
		b—39 (29%)	42	20	9
		c—30 (23%)	44	22	11
Fast neutrons, cyclotron	78	a—56 (72%)	21	6	1
		b—30 (38%)	43	16	3
		c—24 (31%)	46	19	4
detonation (760 rep)	223	a—143 (64%)	25	8	3
		b—66 (34%)	42	18	6
		c—54 (24%)	47	21	8
Control	0	0 (0%)	..	..	..

* a—Based on 2-flower-per-plant sample. b—Based on 4-flower-per-plant sample. c—Based on 16-flower plant sample.

qualitative responses, the authors of the present report observed no external structural aberrations unique for thermal-neutron exposures. The differences detected and recorded here were for pollen lethals.

In conclusion, the relative biological efficiencies of fast neutrons from a nuclear detonation and an equivalent dose from a cyclotron appear to be about 13 or 14 times that of X-rays when mutation rates based on pollen lethals in *Datura* are compared. There is no significant qualitative difference induced by these radiations; the production of chromosomal to gene aberrations is about in the ratio of 4 to 1. A qualitatively different response may be induced by thermal neutrons: the ratio of chromosomal lethals to gene lethals is 3:2.

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A CLOSURE PROBLEM RELATED TO THE RIEMANN ZETA-FUNCTION

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It is rather obvious that any property of the Riemann zeta-function may be expressed in terms of some other property of the function $\rho(x)$ defined as the fractional part of the real number x, i.e., $x = \rho(x) \bmod 1$. This note will deal with a duality of the indicated kind which may be of some interest due to its simplicity in statement and proof. In the sequel, C will denote the linear manifold of functions

$$f(x) = \sum_1^n c_\nu \rho\left(\frac{\theta_\nu}{x}\right), \quad 0 < \theta_\nu \leq 1, \qquad n = 1, 2, \ldots,$$

where the c_ν are constants such that $f(1 + 0) = \sum_1^n c_\nu \theta_\nu = 0$.

THEOREM. *The Riemann zeta-function is free from zeros in the half-plane* $\sigma > 1/p$, $1 < p < \infty$, *if and only if* C *is dense in the space* $L^p(0, 1)$.

Let C^p denote the closure of C in the space $L^p = L^p(0, 1)$, and let T_a, $0 < a \leq 1$, be the operator which takes a function $f(x)$ defined over $(0, 1)$ into the function which is equal to $f(x/a)$ for $0 \leq x \leq a$ and equal to 0 for $a < x \leq 1$. This semigroup of operators has the following properties which will be important for our problem: Each T_a carries C into itself and is norm-diminishing in each space L^p. From this we easily conclude that C *is dense in* L^p *if and only if* C^p *contains the function* k *which is equal to* 1 *over the unit interval.* For, if k belongs to C^p, the same must be true of the characteristic function of any subinterval (a, b) of $(0, 1)$, this function being equal to $T_b k - T_a k$.

We next point out that, for $\sigma > 0$,

$$\int_0^1 \rho\left(\frac{\theta}{x}\right) x^{s-1}\, dx = \frac{\theta}{s-1} - \frac{\theta^s \zeta(s)}{s}. \tag{1}$$

For $f \in C$ we will have

$$\int_0^1 f(x) x^{s-1}\, dx = -\frac{\zeta(s) \sum_1^n c_\nu \theta_\nu^s}{s}, \qquad \sigma > 0. \tag{2}$$

Assume first that $C^p = L^p$. We can then find an $f \in C$ such that $\|1 + f\|_p < \epsilon$, where ϵ is a given positive number. By equation (2),

$$\int_0^1 (1 + f(x))\, x^{s-1}\, dx = \frac{1}{s}\left(1 - \zeta(s) \sum_1^n c_\nu \theta_\nu^{\,s}\right), \qquad \sigma > 0. \quad (3)$$

If $\sigma > 1/p$, Hölder's inequality yields the following majorant of equation (3):

$$\left|\left|1 + f\right|\right|_p \left|\left| x^{s-1} \right|\right|_q < \epsilon \left\{\frac{1}{q(\sigma - 1/p)}\right\}^{1/q}.$$

Consequently,

$$\left|1 - \zeta(s) \sum_1^n c_\nu \theta_\nu^{\,s}\right|^q < \frac{\epsilon^q |s|^q}{q(\sigma - 1/p)}, \qquad \sigma > 1/p. \quad (4)$$

This proves that $\zeta \neq 0$ in the region $D_{p,\epsilon}$ where the right-hand member of relation (4) is < 1, i.e., for

$$\sigma > \frac{1}{p} + \frac{\epsilon^q\ |s|^q}{q}.$$

As $\epsilon \downarrow 0$, $D_{p,\epsilon}$ increases and exhausts the half-plane $\sigma > 1/p$. We should also observe at this instance that $D_{p,\epsilon}$ is convex and that its boundary intersects the line $\sigma = 1$ at the two points where $|s| = 1/\epsilon$.

The proof of the necessity of the condition $C^p = L^p$ is less trivial. If C is not dense in L^p, we know by a classical theorem of F. Riesz and Banach that the dual space L^q must contain a nontrivial element g which is orthogonal to C in the sense that

$$0 = \int_0^1 g(x) f(x)\, dx, \qquad f \epsilon C. \quad (5)$$

Since T_a takes C into itself, it follows that

$$0 = \int_0^1 g(x) T_a f(x)\, dx = a \int_0^1 g(ax) f(x)\, dx, \qquad f \epsilon C, \qquad 0 < a \leq 1. \quad (6)$$

Let E_g^r, $1 \leq r \leq q$, be the closed linear subset of L^r spanned in the topology of that space by the set $\{g(ax),\ 0 < a \leq 1\}$. From equation (6) and the fact that C consists of bounded functions, we conclude that each $f \epsilon C$ is orthogonal to each function belonging to any of the sets E_g^r. We now recall that the positive reals ≤ 1 form a semigroup S under multiplication and that each continuous (and normalized) character of S has the form $\varphi = x^\lambda$, where λ is an arbitrary complex or real number. Clearly $\varphi \epsilon L^q$ if and only if $\Re_e(\lambda) > -1/q$. The problem of whether or not a set of the kind E_g^p contains a character is of considerable complexity. It has been studied earlier by the author[1, 3] and by Nyman.[2] However, the following result[3] can be proved by elementary function theoretic means: *Let $g(x)$ belong to a space $L^q(0, 1)$, $1 < q < \infty$, and have the property*

$$\int_0^x |g(x)|\, dx > 0, \qquad x > 0. \quad (7)$$

Then there exists at least one character x^λ, $\Re_e(\lambda) > -1/q$, which is contained in each set E_g^r for $1 \leq r < q$ (but not necessarily in E_g^q).

In order to apply this theorem, we have first to show that condition (7) is satisfied by our function g. For this pupose assume that g vanishes a.e. on some interval $(0, a)$, $a > 0$. Choose b such that $a < b < \min(1, 2a)$, and $f(x) = b\rho(a/x) -$

$a\rho(b/x)$. This f belongs to C; it vanishes for $x > b$ and takes the value a for $a < x < b$. Therefore,

$$0 = \int_0^1 f(x)g(x)\,dx = a\int_a^b g(x)\,dx, \qquad a < b < \min(1, 2a), \tag{8}$$

and it follows that $g = 0$ a.e. for $x < \min(1, 2a)$. On repeating the same argument a finite number of times, we find that $g = 0$ a.e. over $(0, 1)$, which is a contradiction. The cited theorem may thus be applied to g and yields the existence of a number λ, $\Re_e(\lambda) > -1/q$, such that x^λ is orthogonal to C. On defining $s_0 = 1 + \lambda$, we will have, in particular

$$0 = \int_0^1 \left(\rho\left(\frac{1}{x}\right) - \left(\frac{1}{\theta}\right)\rho\left(\frac{\theta}{x}\right)\right) x^{s_0-1}\,dx = \frac{(\theta^{s_0-1} - 1)}{s_0}\,\zeta(s_0),\ 0 < \theta < 1. \tag{9}$$

Obviously, $s_0 \neq 1$. Since θ can be chosen such that, at any given point $\neq 1$, $\theta^{s-1} - 1 \neq 0$, it follows that s_0 is a zero of ζ. We also have $\Re_e\ (s_0) > 1 - 1/q = 1/p$, and this concludes the proof.

We finally point out that the problem of how well $k = 1$ can be approached by functions $\epsilon\ C$ has a direct bearing on the distribution of the primes even in case ζ does have zeros arbitrary close to the line $\sigma = 1$.

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INTEGRABLE AND SQUARE-INTEGRABLE REPRESENTATIONS OF A SEMISIMPLE LIE GROUP

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Let G be a connected semisimple Lie group. We shall suppose for simplicity that the center of G is finite. Let π be an irreducible unitary representation of G on a Hilbert space $\mathfrak{H}$. We say that π is integrable (square-integrable) if there exists an element $\psi \neq 0$ in $\mathfrak{H}$ such that the function $(\psi, \pi(x)\psi)$ $(x \in G)$ is integrable (square-integrable) on G, with respect to the Haar measure. Assuming that the Haar measure dx has been normalized in some way once for all and that π is square-integrable, we denote by d_π the positive constant given by the relation[1]

$$\int_G |(\psi, \pi(x)\psi)|^2\,dx = \frac{1}{d_\pi},$$

where ψ is any unit vector in $\mathfrak{H}$. Let $C_c^\infty(G)$ denote the set of all complex-valued functions on G which are everywhere indefinitely differentiable and which vanish outside a compact set. Then the following result is an easy consequence of the Schur orthogonality[1] relations.

THEOREM 1. *Let π be an irreducible unitary representation of G on $\mathfrak{H}$ which is square-integrable, and let T_π denote the character[2] of π. Then, if $f \in C_c^\infty(G)$,*

$$T_\pi(f) = d_\pi \int_G dx \left(\int_G f(xyx^{-1})\ (\phi,\ \pi(y)\phi)\ dy\right),$$

where ϕ is any unit vector in $\mathfrak{H}$.

Now suppose G has a Cartan subgroup A which is compact. We extend A to a maximal compact subgroup K. Let $\mathfrak{g}_0$ be the Lie algebra of G and $\mathfrak{g}$ its complexification. We shall assume for simplicity that G has a finite-dimensional faithful representation and therefore there exists a complex analytic group G_c with the Lie algebra $\mathfrak{g}$ such that G is the (real) analytic subgroup of G_c corresponding to $\mathfrak{g}_0$. Let $\mathfrak{h}_0$ and $\mathfrak{k}_0$ be the subalgebras of $\mathfrak{g}_0$ which correspond to A and K, respectively. We define $\mathfrak{p}_0$, $\mathfrak{h}$, $\mathfrak{k}$, $\mathfrak{p}$ as in a previous note[3] and introduce a lexicographic order among roots (of $\mathfrak{g}$ with respect to $\mathfrak{h}$). For any root α we define X_α, $X_{-\alpha}$ and $H_\alpha = [X_\alpha, X_{-\alpha}]$ as before,[3] so that $\alpha(H_\alpha) = 2$. Put $\mathfrak{n}_+ = \sum_{\alpha>0} CX_\alpha$, $\mathfrak{n}^- = \sum_{\alpha>0} CX_{-\alpha}$, where C is the field of complex numbers and α runs over all positive roots. Then $\mathfrak{n}_+$, $\mathfrak{n}_-$ are subalgebras of $\mathfrak{g}$. Let A_c, N_c^+, N_c^- be the complex analytic subgroups of G_c corresponding to $\mathfrak{h}$, $\mathfrak{n}_+$, $\mathfrak{n}_-$, respectively. Then $G_c^0 = N_c^- A_c G$ is an open submanifold of G_c. If ξ is a holomorphic character of A_c, we can choose a (unique) linear function Λ on $\mathfrak{h}$ such that $\xi(\exp H) = e^{\Lambda(H)}$ $(H \in \mathfrak{h})$. We denote by $\mathfrak{H}_\Lambda$ the space of all holomorphic functions ϕ on G_c^0 such that

(i) $\phi(naw) = \xi(a)\phi(w)$ $\qquad (n \in N_c^-,\ a \in A_c,\ w \in G_c^0)$,

(ii) $\|\phi\|^2 = \int_G |\phi(x)|^2\, dx < \infty$.

Then $\mathfrak{H}_\Lambda$ is a Hilbert space under the norm $\|\cdot\|$, and we get a representation π_Λ of G on $\mathfrak{H}_\Lambda$ if we put $(\pi_\Lambda(x)\phi)(y) = \phi(yx)$ $(\phi \in \mathfrak{H}_\Lambda;\ x, y \in G)$. If $\mathfrak{H}_\Lambda \neq \{0\}$, π_Λ is an irreducible unitary representation[4] which is square-integrable. Let 2ρ denote the sum of all positive roots. In case G is simple and not compact, the following four conditions are both necessary and sufficient in order that $\mathfrak{H}_\Lambda \neq \{0\}$:

1. The first Betti number of G is 1.
2. Every noncompact[3] positive root is totally positive.[3]
3. $\Lambda(H_\alpha)$ is a nonnegative integer for every positive compact[3] root α.
4. $\Lambda(H_\beta) + \rho(H_\beta)$ is a negative integer for every positive noncompact[3] root β.

Let $2\rho_+$ denote the sum of all positive noncompact roots. Then, in the presence of the first three conditions, the following condition is sufficient to insure the integrability of π:

4′. $\Lambda(H_\beta) + \rho(H_\beta) < -2\rho_+(H_\beta) + 1$ for every positive noncompact root β.

From now on we shall suppose that G is simple and not compact and that conditions 1 and 2 are fulfilled. We shall also assume that G_c is simply connected. Then it is possible[3] to choose a fundamental system $(\alpha_0, \alpha_1, \ldots, \alpha_l)$ of positive roots such that α_0 is noncompact, while $\alpha_1, \ldots, \alpha_l$ are all compact. Let Λ_i denote the linear function on $\mathfrak{h}$ given by $\Lambda_i(H_{\alpha_j}) = \delta_{ij}$ $(0 \leq i, j \leq l)$. Since G_c is simply connected, there exists a holomorphic character ξ_i of A_c such that $\xi_i(\exp H) = e^{\Lambda_i(H)}$ $(H \in \mathfrak{h},\ 0 \leq i \leq l)$. Moreover, from the theory of finite-dimensional representations of G_c one can deduce the existence of a unique holomorphic function g_i on G_c

such that $g_i(nan') = \xi_i(a)$ $(n \in N_c^-, a \in A_c, n' \in N_c^+)$. Put $g = g_0^{m_0} g_1^{m_1} \ldots g_l^{m_l}$, where $m_0, \ldots, m_l$ are nonnegative integers, and let $g_y(x) = g(xy)$ $(x, y \in G_c)$. Then, if V is the vector space spanned over C by the functions g_y $(y \in G_c)$ and π is the representation of G_c on V given by $(\pi(y)f)(x) = f(xy)$ $(x, y \in G_c, f \in V)$, π is a finite-dimensional irreducible representation of G_c. The corresponding representation of $\mathfrak{g}$ is complex-linear, and its highest weight is $m_0\Lambda_0 + \ldots + m_l\Lambda_l$. On the other hand, suppose that Λ is a linear function on $\mathfrak{h}$ satisfying conditions 3 and 4. Put $\lambda_i = \Lambda(H_{\alpha_i})$ $(0 \leq i \leq l)$. Then $\lambda_0, \ldots, \lambda_l$ are all integers, and $\Lambda = \lambda_0\Lambda_1 + \ldots + \lambda_l\Lambda_l$. Moreover, $\lambda_0 < 0$, while $\lambda_1, \ldots, \lambda_l \geq 0$. Put $g_\Lambda = g_0^{\lambda_0} g_1^{\lambda_1} \ldots g_l^{\lambda_l}$. Then g_Λ is a meromorphic function on G_c. However, it can be shown that g_0 is never zero on G_c^0, and so g_Λ is holomorphic on G_c^0. Also, one can prove that $g_\Lambda \in \mathfrak{H}_\Lambda$, and therefore $\mathfrak{H}_\Lambda$ is the closure of the space spanned by the right translates of g_Λ under G. Thus the analogy with the finite-dimensional case mentioned above is rather close.

Let F be the set of all linear functions Λ on $\mathfrak{h}$ which satisfy conditions 3 and 4. For every $\Lambda \in F$ we have defined a square-integrable representation π_Λ above. Put $d_\Lambda = d_{\pi_\Lambda}$. Then, if m is the number of totally positive roots, we have the following result:

THEOREM 2. *It is possible to normalize the Haar measure of G in such a way that*

$$d_\Lambda = (-1)^m \prod_{\alpha > 0} \left\{ \frac{\Lambda(H_\alpha) + \rho(H_\alpha)}{\rho(H_\alpha)} \right\}$$

for every $\Lambda \in F$.

The analogy with Weyl's formula[5] for the degree of an irreducible finite-dimensional representation in terms of its highest weight is obvious.

Let $\mathfrak{E}$ be the set of all equivalence classes of irreducible unitary representations of G. Let $\mathfrak{E}_0$ denote the subset of $\mathfrak{E}$ consisting of those classes ω which correspond to square-integrable representations. If $\omega \in \mathfrak{E}_0$ and $\pi \in \omega$, we put $d_\omega = d_\pi$. Also, define

$$N_\omega(f) = \left\| \int f(x)\pi(x)\, dx \right\|^2 \qquad (f \in C_c^\infty(G),\ \omega \in \mathfrak{E}),$$

where π is any representation in ω and $\|A\|$ denotes the Hilbert-Schmidt norm[2] of an operator A. It follows from the work of von Neumann[6] and Mautner[7] and a previous result of mine[8] that there exists a *unique* positive measure μ on $\mathfrak{E}$ such that

$$\int_G |f(x)|^2\, dx = \int_{\mathfrak{E}} N\omega(f)\, d\mu$$

for all $f \in C_c^\infty(G)$. On the other hand, one can prove the following result:

THEOREM 3. *The μ-measure of a single point ω in $\mathfrak{E}_0$ is exactly d_ω.*

For any $\Lambda \in F$ let ω_Λ denote the equivalence class of π_Λ. Then $\Lambda \to \omega_\Lambda$ is a one-one mapping of F into $\mathfrak{E}_0$. We denote by $\mathfrak{E}_F$ the image of F under this mapping. It is obvious from Theorems 2 and 3 that we now have an explicit formula for the restriction of the measure μ on $\mathfrak{E}_F$.

[1] See these PROCEEDINGS, **40**, 1076, 1954, Theorem 2; and R. Godement, *Compt. rend. Acad. sci.* (Paris), **225**, 657–659, 1947.

[2] See these PROCEEDINGS, **37**, 366–369, 1951.

[3] See *ibid.*, **40**, 1078, 1954, hereafter cited as "RVI."

[4] See RVI. The last statement of RVI is not correct. In order to rectify it, we have to replace π_ξ there by the definition of π_Λ given above in the present note.

[5] H. Weyl, *Math. Z.*, **24**, 328–395, 1925.

[6] J. von Neumann, *Ann. Math.*, **50**, 401–485, 1949.

[7] F. I. Mautner, *Ann. Math.*, **52**, 528–555, 1950.

[8] *Trans. Am. Math. Soc.*, **75**, 230, 1953, Theorem 7.

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*A FUNDAMENTAL INEQUALITY IN THE THEORY OF PICARD VARIETIES**

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1. *Introduction.*—In abstract algebraic geometry we can define two kinds of irregularities of an algebraic variety, and also one more if the variety is normal. They are, substantially, of different natures, and one of the main theorems in transcendental algebraic geometry asserts that they have the same value if the variety is nonsingular. In this paper we shall prove an inequality among two of them, i.e., we shall show that the dimension q of the Picard variety of a variety V is not greater than the dimension $h^{1,0}$ of the space of linear differential forms of the first kind on V. Actually, we shall prove more, i.e., the following: *Let (A, f) be the Albanese variety of V. Then δf has an "isomorphism property" in the sense that δf gives an isomorphism of the space of linear differential forms of the first kind on A into the corresponding space associated with V.* Since the linear differential forms of the first kind on an Abelian variety are exactly the Maurer-Cartan forms, it follows that the dimension of A, which is equal to q by duality,[1] is not greater than $h^{1,0}$.

2. *Summary of General Results.*—Let f be a function on a variety V with values in a variety U. Let K be a field of definition of f, hence also of V, and let M be a generic point of V over K. If $K(M)$ is separably generated over $K(f(M))$, then f is called *separable.* It can be verified easily that this property is "absolute," i.e., it is independent of the choice of K and M. If the image variety W is not contained in the singular locus of U, we can define a mapping δf of the space of differential forms on U which are "regular along W" into the space of all differential forms on V.[2] The mapping δf is linear over the universal domain $\boldsymbol{K}$ and is commutative with the exterior multiplication and the exterior differentiation of differential forms. Moreover, if ϕ is a numerical function on U which is regular along W, then $\delta f \cdot \phi = \phi \circ f$ holds. Here $\phi \circ f$ denotes the composite function of f and ϕ, which is defined without ambiguity by assumption. Conversely, these properties determine δf uniquely. It is easy to see that f, regarded as a function on V with values in W, is separable if and only if δf gives an isomorphism of the space of all differential forms on W into the corresponding space associated with V. On the other hand, let g be a function on U with values in a variety T. If g is regular along W, and if θ is a differential form on T which is regular along the image variety of g, then we have $\delta f[\delta g \cdot \theta] = \delta(g \circ f) \cdot \theta$. In particular, if f is an inclusion map of V into

U, we have $\mathrm{Tr}_V\,[\delta g\cdot\theta] = \delta(\mathrm{Tr}_V\,g)\cdot\theta$. A differential form on U is said to be *of the first kind* if it is regular at every simple point of any model of the function field of U.[3] If U is nonsingular, the differential form is already of the first kind if it is everywhere regular on U. More generally, if U is nonsingular, δf maps the space of differential forms on U which are everywhere regular on U into the space of differential forms of the first kind on V.[4] In particular, this is always the case if U is an Abelian variety. On an Abelian variety A, a differential form is everywhere regular if and only if it is invariant by δt for all translations t of A. There are as many linearly independent linear differential forms of the first kind on A as its dimension. We know also that every invariant differential form on A is closed, at least if the characteristic of $\boldsymbol{K}$ is different from 2.[5]

3. *The Isomorphism Property of δf.*—Let V be a nonsingular projective model of a function field of dimension 2 over $\boldsymbol{K}$. We have constructed elsewhere[6] a linear pencil $\{C_u\}$ on V with a parameter straight line D of the "simplest possible nature." We have also defined a subvariety $\boldsymbol{J}$ of a product $D \times L^N$ of D and of a projective space L^N such that $\boldsymbol{J}\cdot((u) \times L^N) = (u) \times J_u$ is defined for every (u) and such that J_u is the Jacobian variety of C_u, when C_u is nonsingular, and J_u is a completion of the "generalized Jacobian variety" of C_u, when C_u has a double point. Moreover, we have defined a birational correspondence φ of V into $\boldsymbol{J}$ which has the following property:

LEMMA 1. *The mapping $\delta\varphi$ gives an isomorphism of the space of linear differential forms of the first kind on $\boldsymbol{J}$ onto the corresponding space associated with V.*

On the other hand, let (A, h) be the Albanese variety of $\boldsymbol{J}$. Since h is regular at every simple point of $\boldsymbol{J}$,[7] the composite function $f = h \circ \varphi$ is defined. Moreover, as we have also shown, (A, f) is the Albanese variety of V:

Therefore, by Lemma 1, δf has an isomorphism property if and only if δh has an isomorphism property.

Now let m be a positive integer and consider the m-fold direct product $\boldsymbol{J} \times \ldots \times \boldsymbol{J}$. If $z_1, \ldots, z_m$ are m simple points of $\boldsymbol{J}$, we can define a function H on this product with values in A by $H(z_1 \times \ldots \times z_m) = \sum_{i=1}^{m} h(z_i)$. If we denote by $\boldsymbol{J}_u$ the product $(u) \times J_u$, then a result of Chow can be stated as follows:[8]

LEMMA 2. *Let $(u_1), \ldots, (u_m)$ be independent generic points of D over a field of definition K of $\boldsymbol{J}$, A and h. Then $Tr_{\boldsymbol{J}_{u_1} \times \ldots \times \boldsymbol{J}_{u_m}} H$ is separable for m sufficiently large.*

We shall now show that δh has an isomorphism property. Let θ be a linear differential form of the first kind on A. Then $\delta H\cdot\theta$ is a similar form on $\boldsymbol{J} \times \ldots \times \boldsymbol{J}$. Therefore, if p_i denotes the projection of $\boldsymbol{J} \times \ldots \times \boldsymbol{J}$ to its ith factor, there are uniquely determined m linear differential forms ω_i of the first kind on $\boldsymbol{J}$ such that $\delta H\cdot\theta = \sum_{i=1}^{m} \delta p_i\cdot\omega_i$ holds.[9] Since $\delta H\cdot\theta$ is invariant under the interchange of factors

of the product, the ω_i coincide with each other. Hence we can write $\delta H \cdot \theta = \sum_{i=1}^{m} \delta p_i \cdot \omega$ with a single ω. Here $\omega = 0$ implies $\delta H \cdot \theta = 0$, hence, in particular, $\mathrm{Tr}_{J_{u_1} \times \ldots \times J_{u_m}} [\delta H \cdot \theta] = \delta(\mathrm{Tr}_{J_{u_1} \times \ldots \times J_{u_m}} H) \cdot \theta = 0$. However, since A itself is the image variety of $\mathrm{Tr}_{J_{u_1} \times \ldots \times J_{u_m}} H$, we conclude from Lemma 2 that $\theta = 0$. On the other hand, if we fix $m - 1$ simple points $z_2, \ldots, z_m$ on J, and if z is a variable point on J, we can define a function g on J with values in $J \times \ldots \times J$ by $g(z) = z \times z_2 \times \ldots \times z_m$. Also, let t be the translation of A associated with the point $\sum_{i=2}^{m} h(z_i)$ of A. Since θ is a Maurer-Cartan form, we then get $\delta g[\delta H \cdot \theta] = \delta(H \circ g) \cdot \theta = \delta(t \circ h) \cdot \theta = \delta h[\delta t \cdot \theta] = \delta h \cdot \theta$. On the other hand, we have $\delta g[\sum_{i=1}^{m} \delta p_i \cdot \omega] = \sum_{i=1}^{m} \delta g[\delta p_i \cdot \omega] = \sum_{i=1}^{m} \delta(p_i \circ g) \cdot \omega = \delta(p_1 \circ g) \cdot \omega = \omega$. Hence we get $\omega = \delta h \cdot \theta$, and therefore δh has the isomorphism property.

On the other hand, if V is any algebraic surface, by a result of Zariski together with a recent result of Abhyankar,[10] we can find a nonsingular surface V' which is birationally equivalent to V. Let φ be the function on V with values in V' which represents this birational correspondence. Also, let (A, f') be the Albanese variety of V'. Then $f = f' \circ \varphi$ is defined, and (A, f) is the Albanese variety of V. Since $\delta f'$ has an isomorphism property, δf also has this property.

Finally, let V be an arbitrary variety, and let (A, f) be the Albanese variety of V. In order to prove the isomorphism property of δf, we may assume that $\dim (V) \geq 3$. Let K be a field of definition of A and f, hence also of V, and let L be a generic hyperplane over K in the ambient space of V. Then the intersection product $\bar{V} = V \cdot L$ is defined and is an absolutely irreducible variety of dimension $\dim (V) - 1$ by the "theorem of Bertini."[11] Moreover, if we put $\bar{f} = \mathrm{Tr}_{\bar{V}} f$, then we know that $(A, \bar{f})$ is the Albanese variety of $\bar{V}$.[12] Therefore, if we apply an induction to the dimension of V, we may assume that $\delta \bar{f} = \mathrm{Tr}_{\bar{V}} [\delta f]$ has an isomorphism property. Then, a fortiori, δf has the isomorphism property.

In this way the theorem we have stated in the Introduction is proved completely. We note also that the image by δf of the space of linear differential forms of the first kind on A is composed of closed forms, at least if the characteristic of K is different from 2.

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[1] T. Matsusaka, "Some Theorems on Abelian Varieties," *Natl. Sci. Rept. Ochanomizu University*, **4**, 22–35, 1953.

[2] Cf. S. Koizumi, "On the Differential Forms of the First Kind on Algebraic Varieties. I and II," *J. Math. Soc. Japan*, **1**, 273–280, 1949; **2**, 267–269, 1951.

[3] This definition was given "tentatively" by A. Weil, in *Foundations of Algebraic Geometry* ("Am. Math. Soc. Colloquium Publs.," Vol. **29**, 1946), p. 246.

[4] Koizumi, *op. cit.*, **1**, 277.

[5] S. Nakano, "On Invariant Differential Forms on Group Varieties," *J. Math. Soc. Japan*, **2**, 216–227, 1951. Cf. also C. Chevalley, *Theory of Lie Groups*, Vol. I (Princeton, N.J.: Princeton University Press, 1946).

[6] J. Igusa, "Fibre system of Jacobian varieties," *Am. J. Math.* (to appear).

[7] A. Weil, *Variétés Abéliennes et courbes algébriques* ("Actualités Sci. et Ind.," No. 1064 [Paris: Hermann et Cie, 1948]), p. 27, Theorem 6.

[8] W. L. Chow, "Abelian Varieties over Function Fields," *Trans. Am. Math. Soc.* (to appear); the proof of the result quoted in the text is contained in a separate note, entitled "On Abelian Varieties over Function Fields," which will soon appear in print.

[9] Koizumi, *op. cit.*, **2**, 267.

[10] O. Zariski, "The Reduction of the Singularities of an Algebraic Surface," *Ann. Math.*, **40**, 639–689, 1939; "A Simplified Proof for the Resolution of Singularities of an Algebraic Surface," *ibid.*, **43**, 583–593, 1942; S. Abhyankar, "The Theorem of Local Uniformization on Algebraic Surfaces over Modular Fields," *Ann. Math.* (to appear).

[11] O. Zariski, "Pencils on an Algebraic Variety and a New Proof of a Theorem of Bertini," *Trans. Am. Math. Soc.*, **50**, 48–70, 1941; T. Matsusaka, "The Theorem of Bertini on Linear Systems in Modular Fields," *Kyoto Math. Mem.*, **26**, 51–62, 1950.

[12] Cf. Chow, "Abstract Theory of Picard Varieties," to appear soon in print.

A PATH SPACE AND THE STIEFEL-WHITNEY CLASSES

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The path space described here provides a very simple demonstration of the topological invariance of the Stiefel-Whitney classes for a differentiable manifold M. This invariance has been proved by other means by R. Thom.[1]

Actually, Thom proves a more general result, which implies the topological invariance of these classes. He shows that the fiber homotopy type of the tangent bundle of M depends only on the topological structure of M. Because the Stiefel-Whitney classes are dependent only on the fiber homotopy type of the tangent bundle, their topological invariance follows.

The path space we introduce is regarded as a fiber space[2] over M, and it turns out to have the same fiber homotopy type as the tangent bundle. Since the definition of the path space is purely topological, the general result of Thom follows immediately. Also, one sees directly that the Stiefel-Whitney classes have an analogue for manifolds without differentiability structure.

The paths considered are all continuous parametrized paths in M (parametrized by t, $0 \leq t \leq 1$), which do not recross the starting point (where $t = 0$). So, if $x(t)$ is the point with parameter t,

$$x(t) \neq x(0) \qquad \text{for} \qquad t > 0$$

is the requirement. These paths form a fiber space over M if we define the projection mapping to M by mapping each path into its starting point, $x(0)$.

We can regard M as provided with a smooth Riemannian metric. It is convenient[2] to assume that this metric is such that the geodesic distance between any pair of conjugate points is always more than one. Then, if two points are not more than one unit apart, there is a unique shortest geodesic segment joining them, and this segment varies continuously with the points.

The tangent bundle can now be regarded as formed by the geodesic paths of length 1, parametrized by arc length. This makes it a subspace of our fiber space

of paths. What we prove is that the tangent bundle (thus represented) is a fiber deformation retract of the path space.

The retraction is easy to construct. The essential point is to find a parameter value t^* for each path which varies continuously with the path and has the property that for $0 \leq t \leq t^*$ the points $x(t)$ and $x(0)$ are less than one unit of distance apart. Then a unique shortest geodesic runs from $x(0)$ to $x(t)$.

To define t^*, let $d(t)$ be the distance from $x(t)$ to $x(0)$. Then we define a continuous monotone decreasing function $\delta(t)$ by

$$\delta(t) = \min_{\tau \leq t} |1 - d(\tau)|.$$

This function $\delta(t)$ varies continuously with the path; hence we can define t^* by the equation

$$t^* = \delta(t^*).$$

Using t^*, the deformation of the paths into geodesics of unit length can be defined as a three-stage process. First, contract the path along itself until it becomes the subpath ending at the parameter value t^*. We may now deform it into the geodesic segment from $x(0)$ to $x(t^*)$, with the use of the geodesic segments from $x(0)$ to $x(t), 0 \leq t \leq t^*$. Finally, the geodesic segment from $x(0)$ to $x(t^*)$ is gradually extended until it has unit length.

[1] Réné Thom, *Ann. sci. École norm. supér.* (ser. 3), **69**, 109–182, 1952.

[2] The path space is a true fiber space in the sense that the covering homotopy theorem holds. This fact is actually not needed to justify any of the results we obtain by use of the path space, but it deserves a remark.

One can show by construction that covering homotopy holds locally, even if there is no global differentiability structure on M, so we at least have a fiber space in Hu's sense. But then a recent (unpublished) theorem of Hurewicz shows that this local property implies the global covering homotopy property under very general conditions. Hence the name "fiber space" is justified.

[3] This assumption can be avoided, if desired, by changing the later construction. It is obviously realizable for compact manifolds by scale change and is fairly easily justified in general, so we include no proof of its realizability.

CHANGES IN THE NUCLEI OF DIFFERENTIATING GASTRULA CELLS, AS DEMONSTRATED BY NUCLEAR TRANSPLANTATION

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The hypothesis that the nucleus controls the differentiation of embryonic cells dates back to the time of Roux and Weismann. Originally it involved the assumption of a segregation of nuclear determiners of differentiation during cleavage. When put to the test, the hypothesis was found wanting.[1] Embryological experimentation showed that the distribution of nuclei during the early cleavages could be altered without producing a corresponding alteration of the developmental pattern. Furthermore, cytological evidence of the equational nature of mitosis was

against any theory of differentiation involving somatic segregation. Finally, modifications of the hypothesis which required permanent changes in gene activity were difficult to accept. So far as was known, such changes (i.e., mutations) occur infrequently and unpredictably, while what was required was a co-ordinated series of directed gene changes. Taken together, this evidence indicated that somatic cells contain identical nuclei. However, definite proof of this fact was obtained only for the early cleavage stages, and the possibility remained that nuclear changes, perhaps of a type not heretofore recognized, might occur later in development.[2]

In an effort to detect changes in the properties of nuclei during differentiation, we have developed a method for transferring nuclei of embryonic cells into enucleated eggs of the frog *Rana pipiens*.[3, 4]. The earlier work with this procedure showed that living nuclei of undifferentiated blastula cells could be successfully transplanted. The recipient eggs developed into normal embryos, demonstrating that there had been no irreversible changes in the nuclei during pregastrula development.

More recently, nuclei of chorda-mesoderm and presumptive medullary plate of the late gastrula were similarly tested.[5] The donor cells were small and difficult to handle, and fewer of the attempted transfers were successful than had been the case in the previous experiments. About 8 per cent of the test eggs developed into normal blastulae which, on the basis of their appearance, would have been expected to differentiate normally. Instead of so developing, about half the blastulae were arrested in blastula and gastrula stages. Of the remainder, a few developed into larvae, while the majority were arrested in various embryonic stages. At the time there were two possible interpretations of this result. One was that the nuclei of determined areas of the late gastrula had undergone a change restricting their capacity to promote certain of the varied types of differentiation required for the co-ordinated development of the egg. Such a nuclear change could easily account for the cessation of the development of the test eggs. The second possibility was that the nuclei might have been damaged in the course of the transfer in such a way as to lead to the same result. Since this possibility could not be excluded, we adopted it as the more conservative interpretation and emphasized the fact that some of the test eggs did develop normally. This indicated that at least some, and possibly all, of the late gastrula nuclei were unchanged, despite the fact that the tissues were determined.

Recently the experiments mentioned above have been repeated and extended, using an improved procedure for nuclear transplantation. The improvement consists mainly of the use of trypsin and versene (ethylenediamine tetra acetic acid) to aid in dissecting the embryo and isolating individual cells of known type in undamaged condition.[6] The embryo is first placed in 0.5 per cent commercial (Difco) trypsin made up in a Niu-Twitty solution[7] lacking Ca^{++} and Mg^{++} and buffered at pH 7.1 with phosphate. The trypsin has a preferential action on the material cementing the embryonic layers together. As it acts, the layers can be neatly separated. A portion of the desired layer is now placed in $5 \times 10^{-4}M$ versene, also made up in the modified Niu-Twitty solution. The versene quickly affects the intercellular material—within 5–10 minutes the cells round up and are transferred to the regular Niu-Twitty solution. They can now be picked up singly in the transfer pipette, and the nuclei can be transplanted in the manner previously described.[4]

The improved transplantation procedure was first tested on nuclei of undifferentiated early gastrula cells, with gratifying results (Table 1). The proportion of attempted transfers resulting in normal cleavage of the recipient eggs was increased from 21 to 41 per cent. There was also a pronounced improvement in the later development of the test eggs, as indicated in the table.

TABLE 1

TRANSPLANTABILITY OF NUCLEI AS INFLUENCED BY METHOD OF DONOR CELL ISOLATION
(Donor Cells from Animal Hemisphere of Early Gastrulae)

METHOD OF DONOR CELL ISOLATION	NO. EGGS INJECTED WITH NUCLEI	COMPLETE BLASTULAE	DEVELOPMENT OF BLASTULAE		
			Arrested Blastulae and Gastrulae	Arrested Neurulae and Postneurulae	Larvae (10–12 Mm.)
Dissection with glass needles*	135 (100%)	29 (21%)	10	12	7
Trypsin plus versene	71 (100%)	29 (41%)	1	3	25

NOTE: There is no difference in the type of cleavage that can be correlated with the sources of nuclei. In all cases some nuclear transfers fail completely; others give rise to abortive or "nucleusless" blastulae which never develop further. The remainder, which comprise the genuinely cleaved eggs, give rise to partial or complete blastulae. In this and the following tables we will consider only the formation and development of the complete blastulae.

* T. J. King and R. Briggs, *J. Embryol. Exptl. Morphol.*, **2**, 78, 1954.

The experiments with late gastrula chorda-mesoderm nuclei were now repeated. With the new procedure the proportion of recipient eggs displaying normal cleavage and blastula formation was more than doubled—from 8 to 22 per cent—indicating that more of the nuclei were being transplanted in undamaged condition (Table 2). On this basis one would expect a definite improvement in the later development of the blastulae. However, no such improvement was observed. The majority of the blastulae were still arrested in blastula, gastrula, or abnormal postneurula stages. Sections of the latter embryos showed that the inductor system (notochord and somites) was differentiated, while the central nervous system was very deficient and occasionally absent. These experiments indicated that the deficiencies in the development of the "chorda-mesoderm embryos" were not, as we had previously thought, entirely the result of nuclear damage but rather reflected an intrinsic change in the "differentiative" properties of the chorda-mesoderm nuclei.

TABLE 2

TRANSPLANTABILITY OF NUCLEI AS INFLUENCED BY METHOD OF DONOR CELL ISOLATION
(Donor Cells from Chorda-Mesoderm of Late Gastrulae)

METHOD OF DONOR CELL ISOLATION	NO. EGGS INJECTED WITH NUCLEI	COMPLETE BLASTULAE	DEVELOPMENT OF BLASTULAE		
			Arrested Blastulae and Gastrulae	Arrested Neuralae and Postneurulae	Larvae (10–12 Mm.)
Dissection with glass needles*	242 (100%)	20 (8%)	10	6	4
Trypsin plus versene	83 (100%)	18 (22%)	8	8	2

* See Table 1.

In order to obtain more decisive evidence of nuclear changes during cell differentiation, a series of experiments was done on nuclei of the presumptive mid-gut region of late gastrulae. The cells in this region (floor of archenteron) are large and are easily handled during the transplantation operation, which can therefore be carried out with minimal risk of damage to the nuclei. The results of these experiments showed, first, that the endoderm nuclei elicited normal cleavage and blastula formation in 40 per cent of the test eggs—this being almost exactly the same result as was obtained in control experiments with nuclei of undifferentiated early gas-

trula cells (Table 3). However, the later development of the two groups of blastulae was quite different. Most of the control blastulae developed into larvae, while the "endoderm blastulae" were usually arrested in blastula, gastrula, or abnormal embryonic stages.

TABLE 3

DEVELOPMENT OF EGGS INJECTED WITH ENDODERM NUCLEI OF LATE GASTRULAE

SOURCE OF NUCLEI	No. EGGS INJECTED WITH NUCLEI	COMPLETE BLASTULAE	DEVELOPMENT OF BLASTULAE		
			Arrested Blastulae and Gastrulae	Arrested Neurulae and Postneurulae	Larvae (10–12 Mm.)
Early gastrula (St. 10), animal hemisphere	71 (100%)	29 (41%)	1	3	25
Late gastrula (St. 12), endoderm	67 (100%)	26* (40%)	8	13	5

* There is some variation in the results in the separate experiments. In one experiment 3 out of 11 complete blastulae developed into larvae. In the other two experiments 1 out of 10, and 1 out of 5 blastulae developed into larvae, respectively.

The abnormal embryos were very interesting. They grew to a length of about 6 mm. and displayed a combination of deficiencies which we have not observed in other embryos. This syndrome is characterized by a loss of the integrity of the epidermis, which becomes thickened in some places and thin or absent in others. Internally, the notochord is well developed, somites are large but abnormal in forms and the gut is developed as well as the general condition of the embryo allows. All ectodermal derivatives, however, are very poorly developed and display degenerative nuclear changes. It should be added that these effects on development are not observed when endoderm cytoplasm alone is injected into either enucleated or normally nucleated eggs.

From the results described above it now appears definite that nuclei undergo certain changes during differentiation. These have been best worked out for the endoderm nuclei of late gastrulae. The transplantation tests show that these nuclei have retained the ability to participate normally in cleavage but are usually restricted in their potentiality for differentiation. In some cases chorda-mesoderm is formed, and some attempt at the differentiation of central nervous system and other ectodermal derivatives ensues. In others, development stops sooner, before any neural structures appear. Finally, experiments not reported here reveal that at later developmental stages there is a loss of the capacity of these nuclei to enter into cleavage of egg cytoplasm. All together, this suggests a progressive specialization of nuclear function during cell differentiation. Concerning its nature there is as yet very little known. How it may differ in different tissues, whether it depends upon alterations in chromosomal or other aspects of nuclear function, and the question of correlated changes in cytoplasmic elements are among the problems remaining to be worked out.

A more detailed account of these experiments, and of those involving nuclear transfers from later developmental stages, is in preparation.

Summary.—Nuclei of chorda-mesoderm and endoderm of late gastrulae (*R. pipiens*) were transferred to enucleated eggs. In the successful cases the recipient eggs cleaved normally and developed into complete blastulae, the majority of which were arrested in blastula, gastrula, or abnormal embryonic stages. Evidence is presented to indicate that the failure of the test eggs to differentiate normally is

due to intrinsic restrictions in potentiality for differentiation on the part of the nuclei of the late gastrula.

We are indebted to Miss Marie DiBerardino for her valuable assistance in this work. This investigation was supported in part by a research grant from the National Cancer Institute of the National Institutes of Health, United States Public Health Service, and in part by an institutional grant from the American Cancer Society.

[1] For a review of early literature on this subject see E. B. Wilson, *The Cell in Development and Heredity* (3d ed.; New York: Macmillan Co., 1925).

[2] J. Schultz, *Cancer Research*, **7**, 41, 1947.

[3] R. Briggs and T. J. King, these PROCEEDINGS, **38**, 455, 1952.

[4] R. Briggs and T. J. King, *J. Exptl. Zool.*, **122**, 485, 1953.

[5] T. J. King and R. Briggs, *J. Embryol. Exptl. Morphol.*, **2**, 78, 1954.

[6] P. Rous and F. S. Jones, *J. Exptl. Med.*, **23**, 549, 1916; P. B. Medawar, *Nature*, **148**, 783, 1941; A. Moscona, *Exptl. Cell Research*, **3**, 535, 1952; C. Grobstein, *J. Morphol.*, **93**, 19, 1953; E. C. Slater and K. W. Cleland, *Nature*, **170**, 118, 1952; N. G. Anderson, *Science*, **117**, 627, 1953.

[7] M. C. Niu and V. C. Twitty, these PROCEEDINGS, **39**, 985, 1953.

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